Age and Behaviour Related Changes in Responsiveness to Sensory Stimuli; Effects on Learning and Memory in the Honey Bee, *Apis mellifera*

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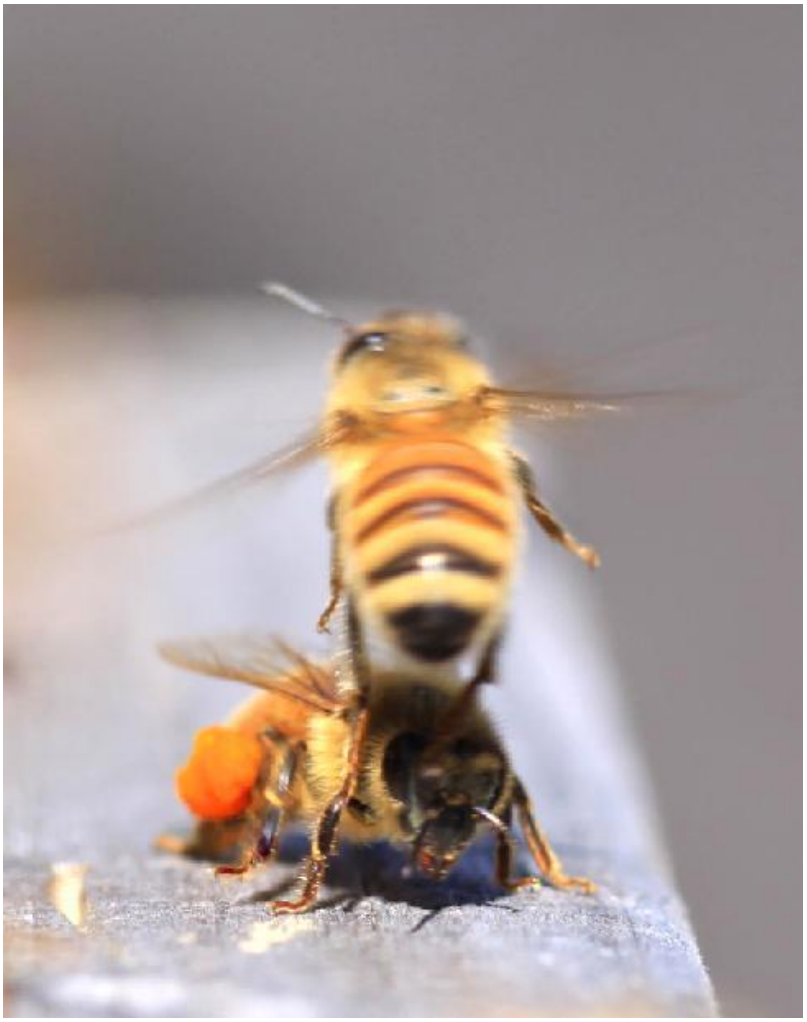

A pollen forager returns as another forager leaves the hive.

Abstract

Behavioural tasks performed by honey bee workers change as they age. These changes in behaviour are suggested in the literature to be influenced by genetics, environmental cues, hormones and biogenic amine levels. In this study age- and performance- related differences in behavioural responses to environmental stimuli were identified in honey bee workers. Guard bees were less responsive to air and odour puffs and habituated more rapidly to mild negative stimuli than young bees (1-6 days old) and pollen foragers. However, guard bees showed a higher sensitivity to aversive stimuli, responding with small sting extensions to low voltages (0.1-1 volts).

One- and 2-day old bees showed faster habituation to tactile stimuli than older bees. Two day old bees also showed poor acquisition of aversive olfactory associations and showed no retention of aversive olfactory memories. However, 2- and 3-day old bees both showed an ability to learn which odour did not pose a threat and they retained this olfactory memory for at least one hour. Guards and pollen foragers successfully learnt aversive olfactory associations and retained aversive memories for 24 hours. Less than half of the pollen foragers examined showed a conditioned response 24 hours after the last conditioning trial, and even fewer bees retained aversive olfactory memory for 48 hours. Pollen foragers collected during winter demonstrated less robust aversive olfactory learning during the acquisition phase but their memory recall was similar to that of summer pollen foragers. The extinction rate of aversive olfactory memories declined faster in guards than in winter and summer pollen foragers. These results indicate that sensory sensitivity, aversive learning behaviour and memory retention change as a honey bee ages or performs different behavioural tasks.

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Index

Figures and Tables

List of Abbreviations

QMP: Queen mandibular pheromone

SER: sting extension reflex (sting extension as an automatic defensive response)

CR: conditioned response (responding to a learnt association)

UR: unconditioned response

CS: conditioned stimulus

US: unconditioned stimulus (the electric shock in aversive learning)

CS+: a conditioned stimulus reinforced with an unconditioned stimulus (e.g. mild electric shock)

CS-: a conditioned stimulus not reinforced

Mild shock: 4 volts (aversive habituation) or 7.5 volts (unconditioned aversive stimulus), 60 Hz, AC current.

1 Introduction

Social Insects

Higher social insects form complex organisations of individuals, or superorganisms (Hölldobler and Wilson, 2009), performing tasks determined by age polyethism, physiology and genetics (Robinson and Page, 1988; Robinson and Page, 1989; Fahrbach and Robinson, 1996; Giray and Robinson, 2004; Behrends and Scheiner, 2009). Eusocial insects that form a social structure include ants, termites, wasps and bees (Wilson, 1971; Oster and Wilson, 1978; Hölldobler and Wilson, 1990; Fahrbach and Robinson, 1995; Hölldobler and Wilson, 2009). These colonial species share a number of features in common. There is generally a single reproductive female, a hundred or more males that are present only during warmer seasons, and thousands of sterile caste workers performing duties in and outside of the colony, caring for the reproductive individuals and their brood (Wilson, 1971; Wilson, 1985; Hölldobler and Wilson, 1990; Hölldobler and Wilson, 2009). Eusocial insects undergo complete metamorphosis during their lifetime. They are capable of flight and show age dependent labour patterns (Rothenbuhler, 1964; Wilson, 1985; Winston, 1987). Communication between members of the colony is conducted via pheromones or through physical contact (antennation) between individuals (Seeley, 1979). Despite these similarities even closely related species show significant behavioural variation. The guards from Africanised and European honey bee colonies, for example, have different response levels to sensory stimuli (Uribe-Rubio and Guzman-Novoa, 2008) and Africanised bees generally show greater aggression toward invaders when irritated.

The Honey Bee

While many social insects are considered to be pests (e.g. termites in wooden homes, wasp or ant nests in our gardens), the European honeybee (*Apis mellifera*) is favoured for its pollination of flowers and its production of honey and wax. Honey is believed to have been harvested by honey gatherers globally since about 13,000 BC. Honey, a high energy source, was originally eaten on the spot, but as people left their nomadic life, swarms of honey bees were caught and managed by bee keepers in man made “hives” for easier access to the stored honey and wax. For over 4,000 years honey bees were kept in clay pots or cylindrical hives

that were destroyed to harvest the honey and wax, killing the colony (Crane, 1983). Over the centuries hive models changed as attempts were made to preserve the colony while harvesting honey, making beekeeping a productive occupation. Several important stepping stones were laid between the 1600s to the 1800s by Swammerdam (Cobb, 2001), Réaumur (Bevan, 1843) and Huber (see, Seeley and Morse 1976) who understood the commercial importance of the honey bee. Their research contributions improved hive design and scientific understanding through observing the colony, mating rituals and studying the anatomy of individual bees. Thomas Wildman's research in the 1700's on traditional and contemporary beekeeping methods (Wildman, 1770) helped Lorenzo Langstroth in the 19th century to design the present day hive (Langstroth, 1852), which was based on spatial distances between the combs described by Huber as 'bee space' (Seeley and Morse, 1976). The Langstroth hive enabled apiarists to extract surplus honey from the comb and return the frame to the hive with minimal disturbance to the queen. Contemporary beekeeping has become an efficient economical method of crop fertilization and a resource for harvesting wax, honey and pollen. Effective husbandry, strong scientific and modern technology has expanded our knowledge about honey bees in the last century. The honeybee is considered an important insect for understanding basic and complex behaviour patterns at individual, cellular and genetic levels (Wilson, 1971; Robinson and Page, 1988; Robinson and Page, 1989; Fahrbach and Robinson, 1996; Menzel, 1999; Menzel and Giurfa, 2001; Giurfa, 2003; Giray and Robinson, 2004; Vergoz et al., 2007a).

Behaviour of the Worker Caste

Age-related changes in behaviour only occur among the worker caste of honey bees (Wilson, 1971). For the first three weeks after emergence the adult worker is normally occupied with tasks within the hive. Newly emerged bees perform cleaning duties around the brood frames. Young bees, 4-13 days of age, generally switch to nursing. Nurses tend to the eggs, feed larvae and, groom and feed the queen and drones (Seeley, 1982; Winston, 1987). Nursing behaviour is triggered by the presence of "brood" and is enhanced by the "perfume" produced by the queen (Robinson, 1992). The queen has a high metabolic demand because of egg production so is fed royal jelly, which contains protein, monosaccharides, fatty acids, vitamins, free amino acids and water (Rembold, 1976). Royal jelly is produced by workers'

hypopharyngeal glands located in the head (Painter and Biesele, 1966). Drones are the reproductive males of the colony. They have a very short proboscis and a large head making it difficult for drones to feed from comb cells unassisted. The drones require workers to feed them; royal jelly, sucrose and pollen granules, while worker bees only consume the latter two foods (Haydak, 1970). At about 14 days of age, workers begin building comb cells, repairing older comb and waxing up gaps around the hive walls. Wax molecules are produced from wax glands on the ventral surface of the abdomen of the workers (Kolmes and Winston, 1988).

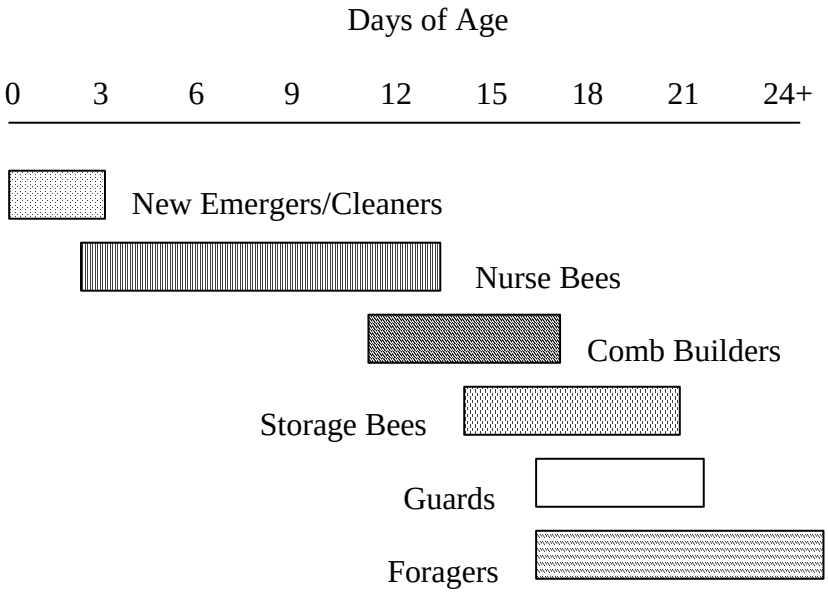


Figure 1.1. Age related changes in behaviour of the worker caste. The movement into a new labour and the length of time performing the task were found to vary between studies. The push and pull factors influencing behavioural changes are determined by genetics, the number of new emergers and the colony needs.

Around the age of 15-21 days old, bees begin attending to food storage, removing bee corpses from the hive and taking up guard duty (Seeley, 1982; Robinson, 1987). Guard duty involves policing the hive entrance to prevent intruders, such as wasps or raiding bees from other colonies, from entering the hive. Guards can be divided into two main groups. The first group consists of bees (usually 10-20 in number) that prevent intruder bees from entering the hive. The second group consists of soldier guards, usually a few hundred that come out of the hive once alerted by alarm pheromone (Moore et al., 1987a). This defensive behaviour is age

dependent and dependent also on sensitivity to alarm pheromones (Collins, 1980). Alarm pheromone is sensed by the bee's olfactory receptors and results in a defensive response to the negative stimuli (Winston, 1987; Balderrama et al., 2002). Colony variation in responses to alarm pheromone is strongly influenced by genetic traits, which also affect the development of guarding behaviour in bees (Lenoir et al., 2006). During the guarding phase bees familiarise themselves with the hive location and the local area by taking orientation flights and exposing themselves to new stimuli. This leads onto a final stage involving foraging. Foraging activity is strongly influenced by social interactions and the depletion of food stores (Lindauer, 1953; Robinson, 1987; Schulz et al., 2002).

A  B 

Figure 1.2. Two nectar foragers (A) and a pollen forager (B) flying back to the hive after foraging in the field for food.

Foraging involves the gathering of pollen, nectar or water to stock food supplies and to maintain a humid atmosphere at a regulated temperature within the hive (Fahrenholz et al., 1989). Foraging is essential for a hive's survival applying high demand on foraging efficiency. New resources are first located by scout bees that inform other bees in the hive of the new resource using the waggle dance and pass on a small sample of the food while advertising the plant's odour cues (Wenner, 1962; von Frisch, 1967; Wenner et al., 1967; Gould, 1974; Tautz, 1996). The waggle dance is used to inform other bees of the location of the resource (von Frisch, 1967). The dance pattern and waggle speed varies depending on which direction and distance the resource is, in respect to the hive (Tautz, 1996; Riley et al., 2005; Thom et al., 2007). Communicating the location of the resource to other foragers increases over time with foraging experience (von Frisch, 1967). Polarised light emitted from the sun is used by foragers to navigate the sky, from their hive to the food resource (Menzel,

1968; Wehner et al., 1975; Rossel et al., 1978). Bees learn to associate specific olfactory and visual cues with nectar quality. Appetitive learning greatly improves foraging efficiency (Menzel, 1968; Cartwright and Collett, 1983; Menzel, 1985).

Age-related changes in behaviour can be influenced by physiological factors, a bee's genetic makeup and the needs of the colony (Robinson and Page, 1988; Robinson and Page, 1989; Fahrbach and Robinson, 1996). A sudden decline in the number of pollen foragers, for example, can increase the number of nurse bees becoming precocious foragers in the colony. An increase in the number of new emergers may also push older nurses off the brood frames and into performing other tasks (Robinson, 1992; Amdam and Omholt, 2003).

Changes in Responsiveness to Environmental Stimuli

Changes in sensitivity to environmental stimuli can trigger changes in task behaviour. Sensory and response differences are thought to be influenced by specific behavioural demands of each labour group (Wilson, 1985; Uribe-Rubio and Guzman-Novoa, 2008). Genetic traits influence behavioural task responses, causing bees of similar age to vary in behaviour, as combinations of patrilines vary between colonies (Lenoir et al., 2006). This is a result of polyandry; the queen mating with several drones (Guzmán-Novoa et al., 2002; Arechavaleta-Velasco et al., 2003; Behrends and Scheiner, 2009). Expression of genes affecting the behaviour of aging bees influences the sensitivity and responses to stimuli (Oldroyd and Thompson, 2006). For example, genes in older bees determine if they forage for nectar or pollen (Robinson and Page, 1989). Therefore, genetics can influence the response to stimuli in bees and the labour they perform. The level of aggressive response or sting extension to a negative stimulus may differ between colonies or nest mates because of genetic variation (Rothenbuhler, 1964; Breed and Rogers, 1991; Behrends and Scheiner, 2009). Evidence implies that responses to environmental stimuli may change with age, influencing movement into another behavioural task. Previous studies suggest that associative olfactory learning and memory may also be affected by age related changes in responsiveness to environmental stimuli (Morgan et al., 1998; Vergoz et al., 2007a; Behrends and Scheiner, 2009).

Learning Development

Olfactory associations begin in the hive where bees first begin to learn to distinguish their kin by odour (Kalmus and Ribbands, 1952; Breed, 1981; Breed, 1983; Getz and Page, 1991). Kinship recognition is important. It enables bees to recognise intruders quickly, and to locate the queen during swarming (Boch and Morse, 1974; Breed, 1981). As bees age, odour recognition becomes important also for foraging. In the early 20th century von Frisch and others demonstrated bees could associate flower cues with a food reward. Bees returned daily to man made feeders at specific times when sucrose solutions were made available and indicated a preference for rewarding feeders over non-rewarding feeders (von Frisch, 1967; Wenner et al., 1967; Waller, 1972; Waddington and Gottlieb, 1990). Menzel and colleagues took advantage of this behaviour to study learning and memory in bees (Frings, 1944; Takeda, 1961; von Frisch, 1967; Bitterman et al., 1983; Menzel and Müller, 1996). Bees quickly learn to extend their proboscis in response to an odour if they have learned to associate the odour with receiving a sucrose reward.

Aversive associations occur naturally also, from a single negative experience, or several encounters. Examples of natural aversive associations include the survival and later avoidance of entrapment in an orb-spinning spider web that imitates a flower (Craig, 1994; Craig and Ebert, 1994) or being hit by violent sex organs of alfalfa flowers (Reinhardt, 1952; Menzel and Müller, 1996). Experienced honey bee foragers will avoid alfalfa flowers or feed on the nectar from the side of the flower. Entering the flower activates the keel mechanism hitting the bee on the head to ensure efficient pollen transfer and cross pollination in the next flower, but operates as a negative stimulus for the honey bee (Reinhardt, 1952). Working the flower from the side enables bees to collect nectar without triggering the keel but the flower does not achieve cross pollination (Reinhardt, 1952). The study of aversive olfactory association in bees is a recent occurrence (Vergoz et al., 2007a). A mild electric shock stimulates the defensive response of a bee (Takeda, 1961; Breed et al., 2004; Vergoz et al., 2007a; Vergoz et al., 2007b) and the bee can quickly learn to associate the presence of an odour with this noxious stimulus.

In recent tests, evidence suggests that 2 day old bees might not be able to associate the presence of an odour with the advent of a negative stimulus (electric shock) (Vergoz, 2008). In contrast, Vergoz found that 4 day olds showed aversive olfactory learning and

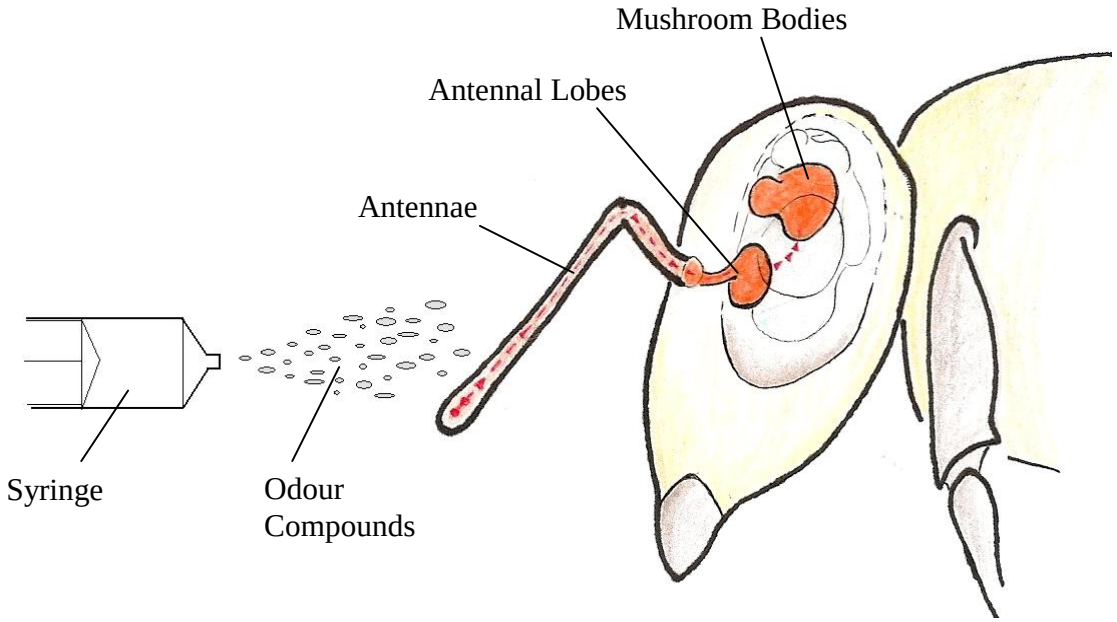


Figure 1.3. Odour compounds are detected by receptors in the antennae. The signals are carried via the antennal nerve to the antennal lobes. Information processed in the antennal lobes is then conveyed to the mushroom bodies of the bee's brain. The mushroom bodies are involved with olfactory learning and memory.

retained aversive olfactory memories for at least 1 hour (Vergoz et al., 2007b). These experiments suggest that aversive olfactory learning and the formation of aversive memories might be age dependent. Aversive learning and memory formation in 4 day olds was only possible when QMP (queen mandibular pheromone) was not present after emergence. QMP is a multifunctional pheromone produced in the queen's mandibular glands (Slessor et al., 1988). Its purpose is to advertise her presence, health, and maintain behavioural control over the colony. QMP affects the physiology and behaviour of the worker bees by reducing ovary development (Winston et al., 1990) that would otherwise begin laying unfertilized eggs in a queen-less hive (Tamer et al., 2006). QMP encourages other hive activities like comb building (Ledoux et al., 2001) but reduces locomotor activity and suppresses aversive learning and memory of younger bees (Vergoz et al., 2007b). Previous experiments, showed bees raised with QMP only began responding to an odour that had been conditioned with an aversive stimuli by the age of 15 days (Vergoz et al., 2007a). One week old bees were not

able to learn or retain aversive associations when exposed to QMP, but they did show significant learning ability at 4 days and older when not raised with QMP (Vergoz et al., 2007a). The pheromone reduces dopamine levels in the brain and influences dopamine signalling (Beggs et al., 2007). If the dopaminergic neurons within the brain are blocked the insect's ability to form aversive associations is greatly reduced as dopamine is essential for aversive learning (Schwaerzel et al., 2003; Unoki et al., 2005; Vergoz et al., 2007a).

This study will determine (i) whether sensitivity to puffs of air, odour puffs, and mild aversive stimuli changes with age, (ii) how quickly responses to odour and mild aversive stimuli habituate and whether this is age dependent, (iii) the age at which aversive learning in worker bees can first be demonstrated, (iv) whether long-term aversive memories are established in pollen foragers and guard bees, (v) whether seasonal change influences aversive learning in pollen foragers, and (vi) how quickly conditioned responses can be extinguished. The overall aim is to develop a better understanding of age-related changes in sensory responsiveness, and the development of aversive learning in bees.

2 Methods

2.1 Collecting worker bees

To identify bees of known age, newly emerged adults were collected from brood frames (Fig. 2.1.A). Frames were taken out of the hives located at the Department of Zoology and placed into incubators set at 36˚C. Bees that emerged within a few hours of each other had their thorax colour coded with paint (water based, non-toxic acrylic). The paint was allowed to dry and the bees were returned into their parent hive (Fig. 2.1.B). The bees were collected again from the hives when they were at the required age. To prevent exposure to QMP some newly emerged bees were kept in cages in the laboratory from the time of adult emergence until they were 2 or 3 days old. They were supplied 30% sucrose solution and water via Eppendorf tubes. Collection of guard bees (Fig. 2.1.C) was done by selecting bees policing the entrance and by provoking the hive entrance with a stick. The bees that exited the hive within the first minute and remained on the entrance platform investigating the stick were considered to be soldier bees, the second group of guard bees (Breed et al., 1990).

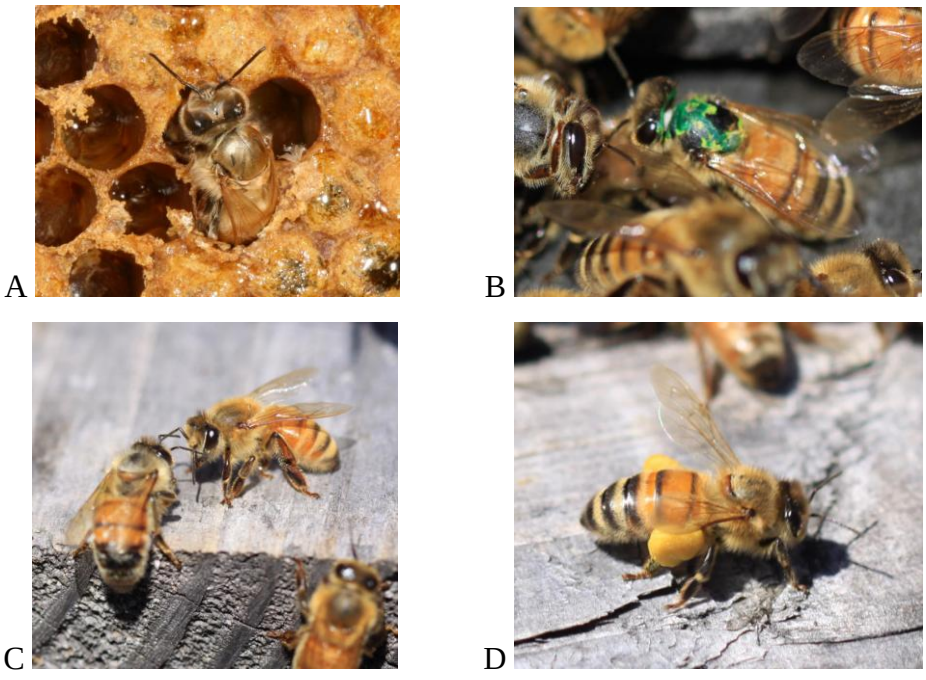


Figure 2.1. A newly emerged bee crawls out of her cell after chewing off the wax capping (A). A painted bee of known age works amongst her sisters inside the hive (B). Guard bees outside the hive entrance police all bees arriving (C). A pollen forager returning with her pollen baskets fully loaded (D).

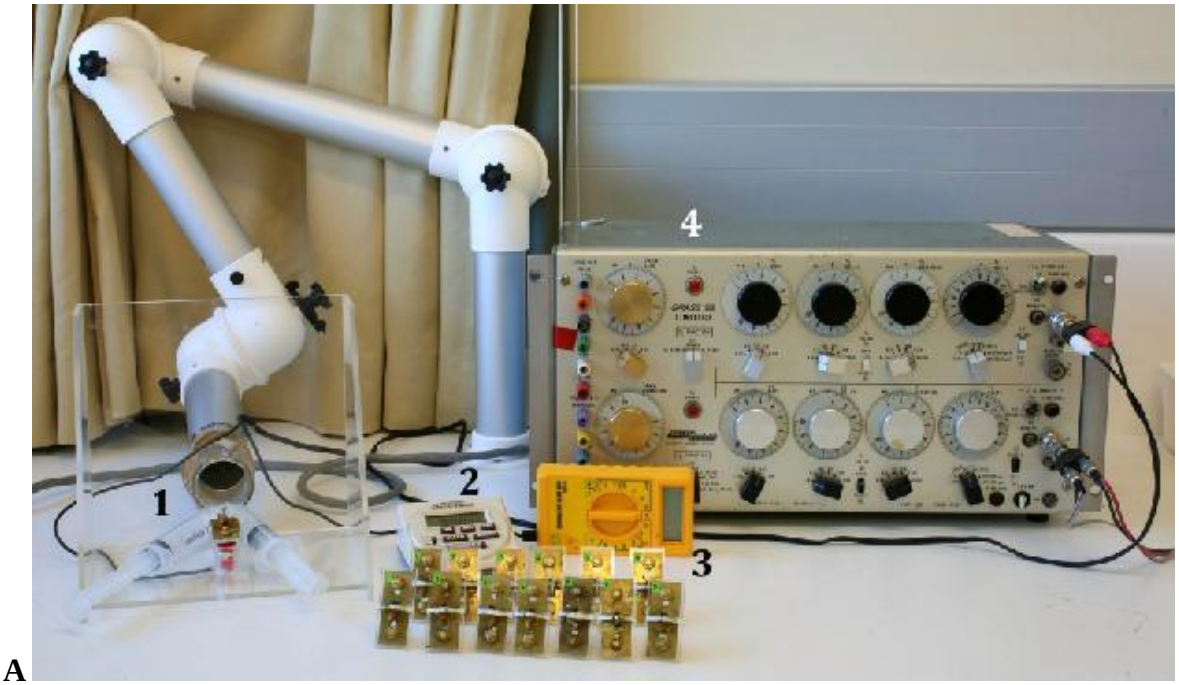
Pollen foragers (Fig. 2.1.D) were collected from the hive entrance as they returned from foraging. These bees are easily distinguished from others by the large pollen granules on their hind legs.

2.2 Experimental Setup

Bees of the following ages from adult emergence were examined: 1, 2, 3, 4 and 6 day olds. Guard bees and pollen foragers (>15 days old) were also examined. Only 20 bees were collected at one time. All experiments required bees to be restrained in holding frames. The same process of harnessing the bees was used at the beginning of all experiments. Firstly bees were cooled down inside a glass jar covered with ice until they become stationary. They were then placed onto a holding frame with their thorax between two brass plates (Figure. 2.2.C). The bee's neck and petiole were fitted into small groves cut into the brass plates. A conductive gel, electrophysiology cream (Reegraph M.E.I) was used to improve electrical conductivity between the plates and the bee's body. A thin strip of tape was wrapped over the thorax to ensure the bee could not escape but had free movement of the head, antennae and abdomen. Bees were left for one hour to become accustomed to the holding apparatus and the unusual position. After 20 minutes into the hour bees were fed honey to replenish their energy and to obtain better results. At the beginning of each experiment bees were placed into the training arena (Figure. 2.2.B). The holder in which the bee was placed was connected to a stimulator that was used to administer electrical stimuli of known voltage to the bee (Figure 2.2.A).

2.3 General responsiveness to sensory stimuli

A series of tests were carried out to determine whether there are age-related changes in responsiveness to stimuli, such as a puff of air or odour and a mild electric shock.
The bee was exposed to a puff of air or odour from a 20 ml syringe. The odour puff was formed by placing 5µl of eugenol (Sigma) onto a small piece of filter paper (1 cm^2) which was then placed into the syringe. The syringe was held about 1cm from the bee's antennae and the puff was delivered for 5 seconds. The abdomen was observed for sting extension during and directly after the puff was administered. The bee's response to the stimulus was recorded.



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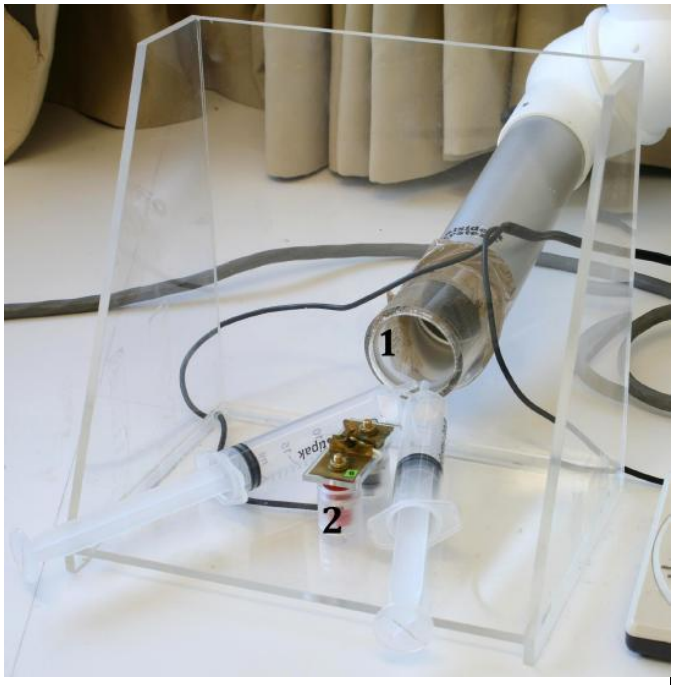

B

C

Figure 2.2. (A) The apparatus setup used to test sensitivity to shock stimuli and for aversive learning. Shock duration and voltage was controlled by the Grass SD9 stimulator (4). A voltmeter (3) was used to double check the voltage being delivered to the bee (1). The shock duration was timed using a stop watch (2) to ensure all bees received the correct exposure length.
(B) Training arena. An air extractor (4) was used to remove odour from the arena. The stage in the arena centre (2) was connected to the stimulator used to deliver a shock stimuli.
(C) A bee secured in a holder between two brass plates.

To avoid using an odour containing a key odour cue previously experienced (Bruce et al., 2002; Vereecken et al., 2010), eugenol, single compound odour, was used. Floral odours contain compound mixtures, some compounds are stronger than others and are more likely to become a key triggering component in a conditioned stimulus (Deisig et al., 2010). A novel odour containing a pre-experienced key compound could trigger a reflex response to the previous associations.

The voltage required to elicit sting extension (the response threshold; Figure 2.3.) was determined in bees of different ages by exposing bees to voltages ranging from 0.1 to 8 volts. The voltages tested were similar to those used by Roussel *et al*. (2009). The maximum voltage administered in the present study was 8 volts as previous studies have observed abnormal behaviour occurring in bees exposed to higher voltages (Vergoz, 2004) and because approximately 90% of foragers exhibited a sting extension reflex at 8 volts. To determine the response threshold the voltage was set at 0.1 volts and increased every two minutes to 0.25, 0.5, 1, 2, 4, 8 V as described by Roussel *et al*. (2009). The bee's abdomen was observed before, during and after administering each shock stimulus but only the response during stimulation was recorded. Five possible responses could occur; full sting extension, partial sting extension, small or large body movements or no response. This experiment highlights the changing threshold to aversive stimuli with age.

Figure 2.3. Large sting extension in response to stimulation of 8 volts.

2.4 Non-Associative Learning: Habituation

Rates of response habituation (non-associative learning) were examined in bees of specified ages. The stimuli tested included odour puffs and mild (4v) shock stimuli of 2 seconds duration. Each bee was exposed to one stimulus 15 times with a 2 minute interval between each stimulus. The bee's defensive response (sting extension) was recorded. The aversive stimulus of 4 volts was well above the threshold for large sting extension threshold but not strong enough to cause physical harm. The rate of habituation to an odour puff given to pollen foragers was also tested and compared to response curves obtained for the unpaired stimulus (CS−) in differential conditioning trials (See section 2.6).

2.5 Associative Olfactory Learning

Associative olfactory learning was demonstrated using absolute conditioning as well as differential conditioning using techniques similar to those described by Vergoz et al. (2007 a,b). Absolute conditioning involves conditioning with one odour which is paired with an electric shock. Differential conditioning involves two odours one of which is reinforced with mild shock (CS+) and the other is not reinforced (CS-).

2.5.1 Absolute Conditioning in 2 Day Old Bees

The odour eugenol was paired with a mild electric shock of 7.5 volts and 2s duration (Fig 2.5). Six conditioning trials were used with 10 minute intervals between each conditioning trial. A retention test was preformed one hour after the last conditioning trial (Fig 2.4). The aim of this experiment was to confirm findings of Vergoz (2008) who showed that 2 day old bees show little or no aversive learning.

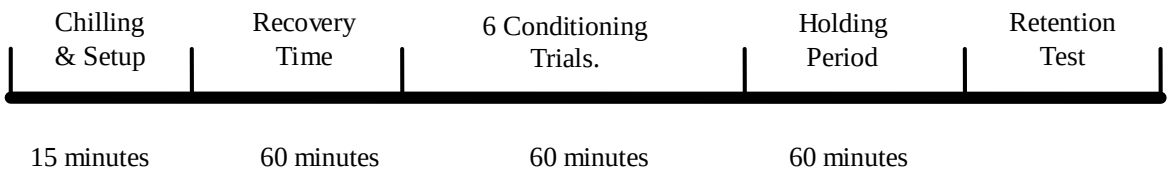

Figure 2.4. Absolute conditioning timeline.

2.5.2 Differential Conditioning

Each bee was placed into the training arena (see section 2.2.) and left for 20 seconds to adjust to the new environment. Next, one of two odours, eugenol or 2-hexanol, was delivered to the bee for 5 seconds. For the purposes of these experiments eugenol (CS+) was paired with the unconditioned stimulus, mild electric shock (US), whereas 2-hexanol (CS-) was not reinforced. The CS+ odour was puffed towards the bee for 3 seconds alone and then together with the US, a mild shock (7.5 volts, 60 Hz, AC current) for a further 2 seconds (Fig 2.5.).

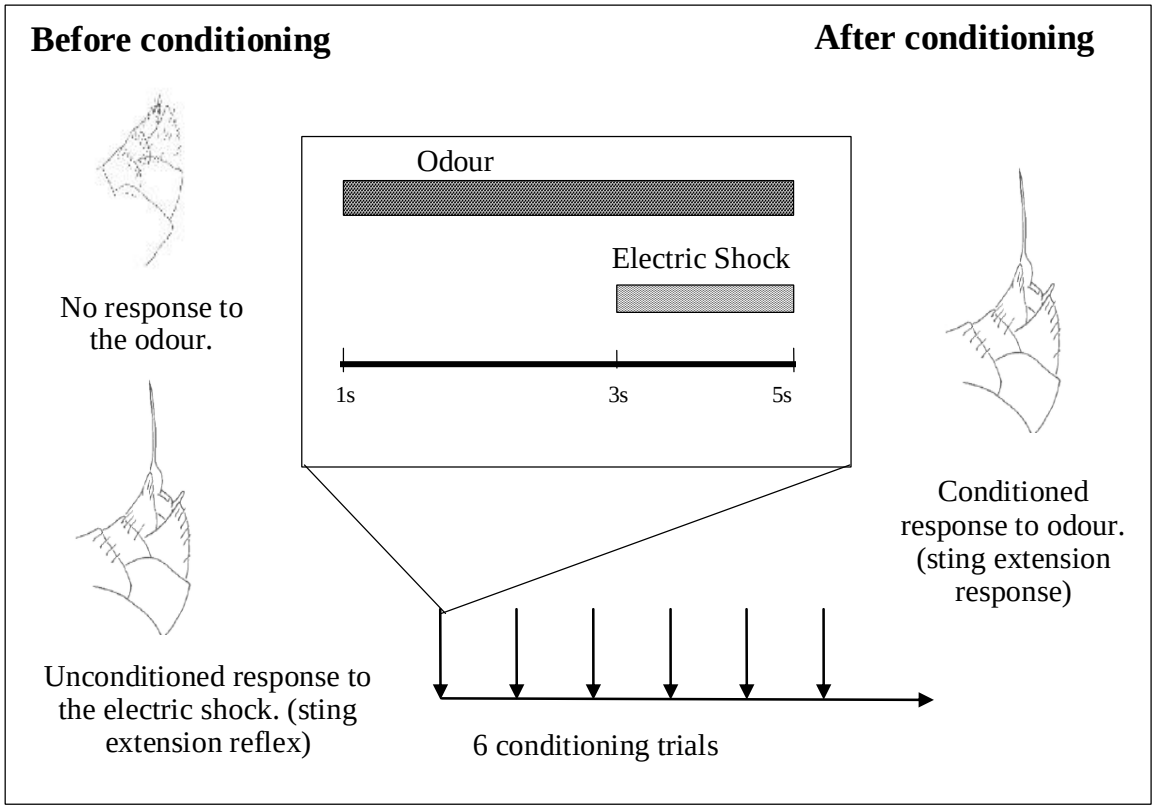

Figure 2.5. Protocol used for aversive conditioning. Prior to conditioning no sting extension occurs in response to the odour. The unconditioned response to the electric shock is a large sting extension. In each trial the odour is presented for three seconds then continued simultaneously with a 2 second mild electric shock (7.5 volts). Bees were conditioned 6 times to an odour (arrows). If a bee learns to associate the odour with punishment, she will extend her sting to the odour in anticipation of the shock. Modified from Vergoz, 2008.

Conditioning was repeated 6 times for each odour, with a 10 minute interval between each trial (Fig. 2.5). The two odours were presented in an organised random order (A, B, A, B, A, A, B, B, A, B, A, B) to ensure no pattern of odour exposure was learned. After conditioning, bees were placed in an upright position for 1, 24 or 48 hours after which time the retention test was performed. During the retention test bees were exposed to CS+ (alone), and to CS-, each for 5 seconds. A sting extension during exposure to the conditioned odour (CS+) indicates that the bee has learnt to associate the odour with punishment. No sting extension to CS- helps confirm that the bee is able to distinguish between the two odours. Responses to the US were retested also to confirm that the bee still showed a normal reflex response.

Differential conditioning was used to examine aversive learning in 3 day old adults that had not been exposed to QMP. As bees at this age showed signs of learning and memory retention, 2 day old adults were tested also to determine at what age bees first exhibit aversive learning.

2.5.3 Retention Duration

Short term memory (1 hour) following aversive learning has been demonstrated (Vergoz et al., 2007a; Vergoz et al., 2007b; Roussel et al., 2009) but it is not known how long aversive memories are retained beyond this time point. Pollen foragers and guard bees were used to determine whether aversive memories are retained for longer periods. The aversive learning procedure followed the same techniques as described above. However, after the final conditioning trial, bees were placed into an incubator over night while still restrained in their holding frames (Fig. 2.6). Bees were fed honey regularly to ensure survival. Retention testing followed 24 or 48 hours after conditioning.

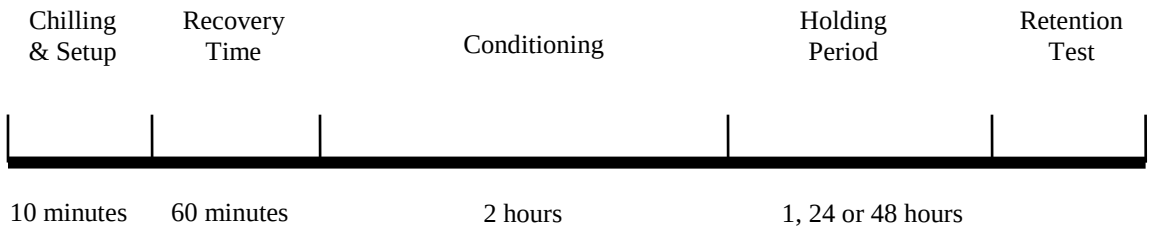

Figure 2.6. Timeline for differential conditioning to CS+ and CS- odours.

2.5.4 Extinction of Conditioned Responses

The following experiment was aimed to determine how quickly the conditioned response towards CS+ odour can be extinguished if bees had elected a sting response during retention testing. After the retention test (see section 2.5.3), bees were presented with the conditioned stimulus for 5 seconds without reinforcement for 15 trials at 2 minute intervals. Any sized sting during or directly after exposure was counted as a response to the odour. Results indicated how fast the bee is able to extinguish the conditioned association when reinforcement is not present.

2.5.5 Responses to CS-

During aversive learning, responses to CS- fell from 20-30% down to about 5-0%. To determine if this rate of decline is influenced by presentation of CS+, two groups of pollen foragers were presented with 2-hexanol alone, without reinforcement for 6 trials. The odour was presented at 10 or 20 minute intervals for 5 seconds. A sting extension was considered as a response to the odour. This experiment aimed to determine whether the decline in response to a neutral stimulus varies depending on how frequently it is received, and if another stimulus (CS+) is present for comparison.

3 Statistical Analysis

Data were analysed using SPSS 17, SAS (Statistical Analysis Software version 9.1, Institute Inc. Cary, NC, USA) and Minitab 15.

3.1 Responses to a novel stimulus

One-way ANOVA was used to compare the mean level of responses to a novel stimulus (an air puff or odour puff) across all age groups. This gave an F statistic, a ratio of variance between the means, post hoc comparison (Turkeys test). Two sample t-tests were used to compare mean responses towards an air puff versus of an odour puff response in each group.

3.2 Response changes resulting from repeated stimulation: Habituation

Habituation of sting extension in response to repetitive exposures to the same stimulus (either an odour puff or a 4 volt electric shock stimulus) was tested using several different methods of analysis. Bees that did not respond with an extended stinger in the first trial were excluded from the data. The exclusion compensated for the possibility some bees were not physically able to sting or sense the stimulus.

3.2.1 Habituation to repeated odour puffs

To examine changes in response levels within repeated stimulations non-linear regression was used. Parameter estimates of the mean and standard deviation were then used to compare groups using two-sample t-tests. The R^2 value was calculated by using the model expression of (a + b*exp(-k * Trial)) which indicates how closely the data points fit the predicted curve. The numerical distance of the R^2 value to 1 (predicted curve) indicates the degree of variation of the response levels from the predicted curve. T-tests were conducted using the standard error of the difference between angular coefficients to gauge if differences between any of the three groups were statistically significant. This enabled us to determine if the regression curve in one group is different from the regression curve of another group.

3.2.2 Response to a repetitive negative stimulus

The changes in response levels and large response fluctuations meant non-linear regression could not be used to analyse the 4 groups. The rate of habituation to 4 volts every 2 minutes was analysed first using a Chi-Squared test to compare the percentage of sting extension reflexes occurring in the final trial across the 4 groups. After a significant variation in the level of responses across groups was confirmed, a generalised linear model was used to determine where the significant differences occurred between the groups last response levels. Generalised linear models are a flexible overview of ordinary least squares regression using binomial data.

3.3 Behavioural Threshold Limits

To form clarity from the low level behavioural responses to the increasing voltages a response threshold was formed. The two sting extension responses from the first three voltages administered to the bees were used to calculate X (the mean) and multiplied by 100. A statistical equation taken from binomial distributions was used ($L_2=((X+1)\ F\alpha(2),v1'v2')/(n-X+(X+1)\ F\alpha(2),v1'v2')$) to calculate the upper confidence limits for proportions (Zar, 1984). The final result of L_2 is multiplied by 100 and was used as the upper limit (behavioural threshold). The calculated upper limit is used as a method to cancel out any 'noise' that occurs randomly. The minimum voltage required to elicit a response above this threshold level was recorded. A linear mixed model calculated the F-value from the change in behavioural response levels corresponding to voltage change while the difference of least squares means give the sidak values (a multiple comparison amongst a set of means) of every interaction between the voltage and behavioural response patterns. These analyses show that different behavioural patterns form in correlation to the voltages and the degree of difference.

A Pearson Chi-Squared test was used to verify variations of small or large sting extension percentages at voltage points of interest between bee groups. Significant results were investigated using a pairwise comparison from a generalised linear model to find exactly which group was significantly different from other groups of bees.

3.4 Absolute and Differential Conditioning and Retention

To determine whether the level of responses to CS+ (Fig 3. A, B see a,a.i) and CS- (Fig 3. A, B see b,b.i) change significantly over the 6 conditioning trials Cochran tests were used. The Cochran test is a non parametric statistical test which can be used to analyse dichotomous data making it ideal for analysing changes in the acquisition curve (Zar, 1984). Cochran testing will determine if a significant change occurs in the level of conditioned responses over the six trials for CS+ or CS- within a group.

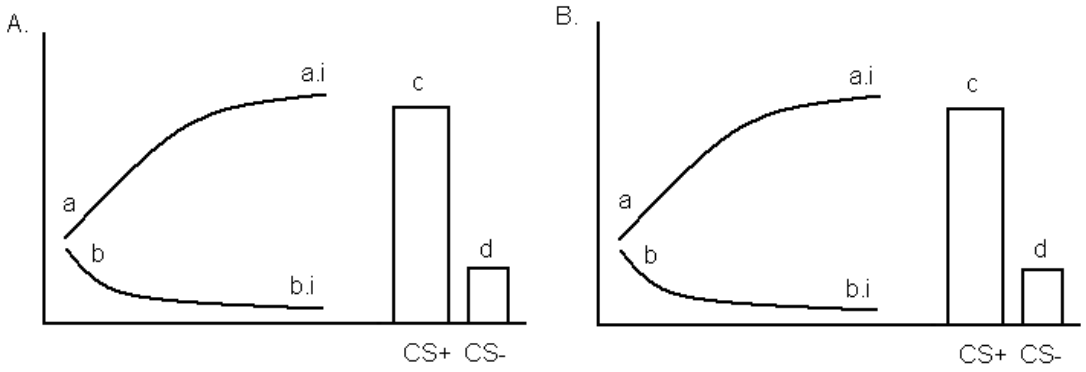

Figure 3.1. An example diagram of differential conditioning and retention test results from two separate groups of bees, A. (left) and B. (right). a to a.i: conditioned sting extension response levels to CS+ odour paired with an electric shock. b to b.i: conditioned sting extension response levels to CS- odour unpaired. c: responsive sting to the CS+ odour during retention testing not accompanied with a shock. d: responsive sting towards CS- odour.

The McNemar test is a modified Chi-squared test for within group comparisons of paired dichotomous data (Zar, 1984). The McNemar test is used to determine whether response levels in the very first conditioning trial (prior to electric shock treatment) differ significantly from the level of response observed in the retention test for CS+ and CS- (Fig 3. A, B. see a and c, b and d). The reason for using the first conditioning trial is that this represents the spontaneous response level prior to presenting the animal with an aversive stimulus. To compare the level of responses to CS+ and CS- in the retention test (Fig 3. c and d) a McNemar test is also used. This analysis will show if bees have learned to associate the CS+ odour with punishment.

Chi-Squared tests were used to compare the level of responses recorded in the retention tests between two different bee groups e.g. (Fig 3. A.c vs B.c). Comparing the

corresponding retention test response levels to CS+ or CS- with another bee group shows the level of difference in memory retention.

To compare the response levels across the conditioning trials between two groups the delta value is calculated first by (CS+)-(CS-). The new data points at each conditioning trial are compared with the same trial number from another bee group using a Mann-Whitney test. It is a non-parametric test for analysing if two levels of response are the same. In the conditioning trails it is used to determine if calculated sum responses to the two odours are different to those recorded in another bee group. The same analysis also assessed response levels in the retention test among CS+ and CS- between bee groups.

3.5 Extinction Rate

Bees that displayed a conditioned response to the reinforced odour (CS+) in the retention test were used to determine how quickly the memory could be extinguished by presenting the odour without reinforcement. ANOVA residual tests gave an R^2 value that correlates the variation of each data point at every trial from the best fit line calculated from the original data. Next, groups were compared in a pairwise manner using two sample t-tests to find out which groups were significantly different from one another. A significant result shows the rate of response level decline to the odour from one group of bees varied from another and was able to extinguish prior associations faster.

3.6 Changes in response to CS-

Changes in the level of responses to the non-reinforced odour (CS-) were analysed in three ways. Firstly a Cochran test analysed the change in response levels over 6 trials to determine if the change of response levels over the 6 conditioning trials were significant. Secondly a linear regression, a linear line estimated from the data, is formed using a non-standardised coefficient on the slopes to calculate the R^2 value. Thirdly, changes in the level of responses elicited from all bee groups tested were compared between groups of interest using a general linear model, univariate analysis of variance. The model calculates an estimated curve and compares the two slopes. A significant difference between the groups indicates the decline of response levels between them were not the same over the 6 trials.

4 Results

4.1 Age-related Changes in Responsiveness to Air and Odour Puffs

4.1.1 Levels of Responses to a Novel Stimulus

The percentage of bees displaying sting extension in response to a novel stimulus, a puff of air (Fig 4.1 grey bars) or an odour puff (Fig 4.1 white bars) to the antennae, was recorded using 1-6 day olds, guards and pollen foragers. Significant differences were found between the responses of bees of different ages to an air puff (1 factor ANOVA, $F._{5,376}= 3.63$, $p=0.003$) and to an odour puff (1 factor ANOVA, $F._{5,379}= 4.47$, $p=0.001$). Four day old bees showed the highest level of responses to both stimuli. Responses tended to increase from 1 day to 4 days old and then declined.

The level of responses to an air puff in 4 day old bees was significantly higher than the level received in 6 day olds, guards and forager bees (Fig. 4.1). Guard bees had the lowest response level (12.12%). The level of responses to an odour puff remained relatively

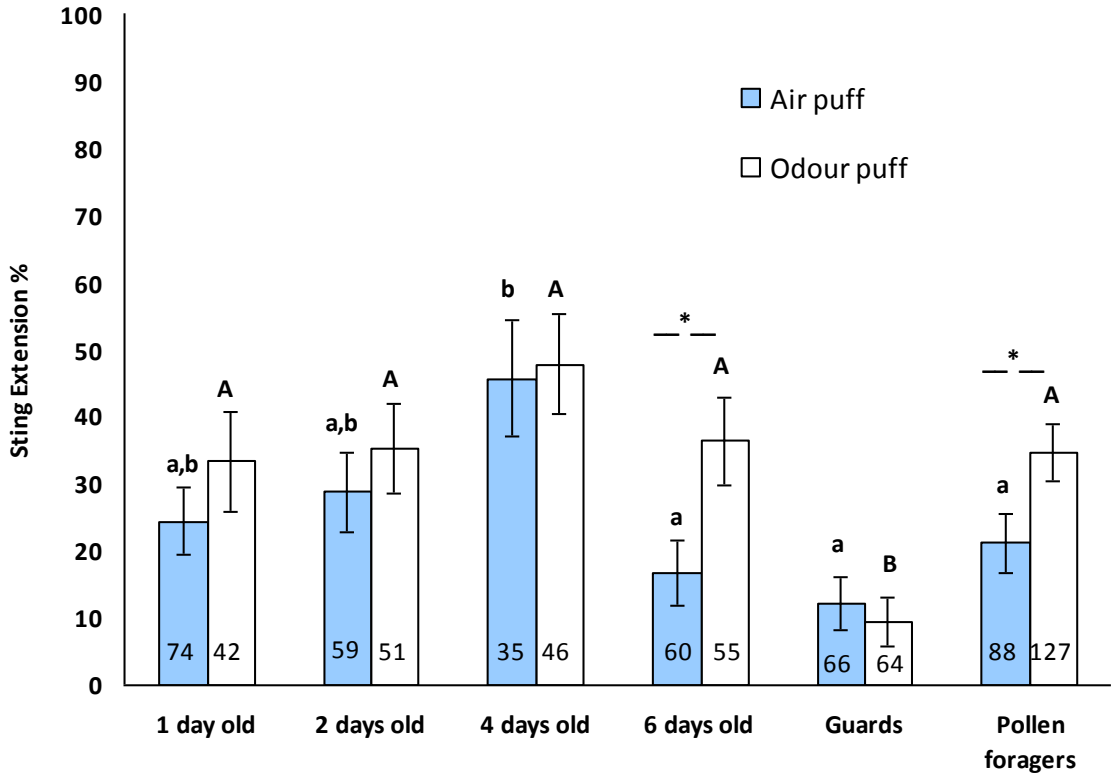

Figure 4.1. Percentage of bees responding with sting extension when either air or an odour was puffed at their antennae.

high in 6 day old bees and pollen foragers but the level of responses to an odour puff was significantly lower in guard bees than in all other groups.

The levels of responses towards the two stimuli within a group were significantly different for 6 day olds (two-sample t-test, t-value = -2.42, DF = 101, p = 0.017) and pollen foragers (two-sample t-test, t-value = -2.34, DF = 204, p = 0.02). Responses to the two stimuli within the other groups were similar to each other with responses to odour puffs always being slightly higher except in guard bees.

4.1.2 Habituation to Odour Puffs

All groups showed a strong correlation between response levels and trial number, with responses declining as a result of repeated stimulation (Fig. 4.2.; R^2: 1 & 2 day olds = 0.977; 4 & 6 day olds = 0.945; Pollen foragers = 0.977). Most bees habituate to the odour stimulus. However two sample t-tests revealed significant differences between the groups; 1 and 2 day old bees habituated faster in the first two trials than the other two groups (1 & 2 day olds versus 4 & 6 day olds: T-value = 16.64, DF = 27, p = 0.0001; 1 & 2 day olds versus pollen foragers T-test = 20.55, DF = 23, p = 0.0001). A small percentage of pollen foragers continued to respond even in the 15th trial.

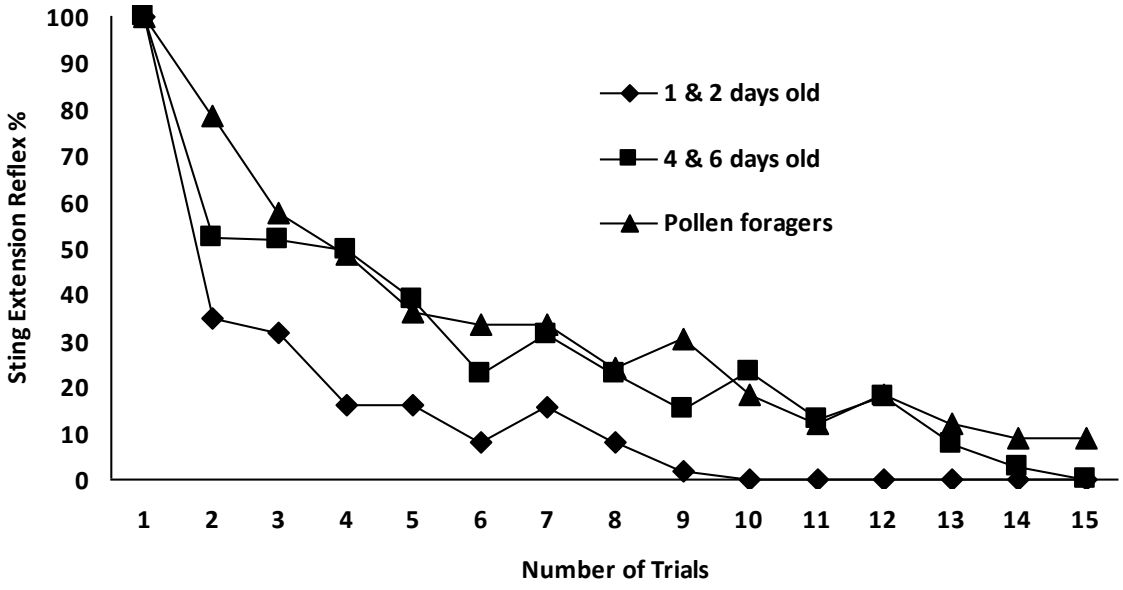

Figure 4.2. Habituation of the sting extension reflex in response to odour puffs delivered every two minutes, 15 times. (1 & 2 days n = 34; 4 & 6 days n = 28; Pollen foragers n = 33).

4.2 Responses to Aversive Stimuli

4.2.1 Response Thresholds

Behavioural responses towards electric shock stimuli at a range of voltages were tested in bees of different ages. At the lowest voltages tested (0.1-0.5 volts) only 0 to 5% of bees responded. To deal with this 'noise' a threshold line was calculated from the upper confidence limit of the average 'noise' level for sting extension responses. All behavioural response levels above this line are considered to be above threshold for any particular behaviour.

The threshold line for 1 day old bees was calculated to be 10.2% and small movements were already higher than this with 27% at 0.1V (Fig. 4.3.). All other behavioural responses did not pass the threshold level until 2 volts. Small movements decreased by 4 volts, while small sting extension responses peaked at 4 volts and dropped slightly by 8 volts. Only large sting extension continued to increase with voltages above 4 volts indicating that it is the most prominent response when a bee is harmed. The fixed effects comparison of the behavioural response levels with voltage increase shows a highly significant difference between the response changes as voltages increase ($F_{18,1536} = 24.48$, $p = 0.0001$).

In 2 day olds small movements began slightly above the threshold line (16.9%) (Fig 4.4). Large body movements and small sting extension remained under the threshold limit until 2 volts. Large movements declined with voltages above 2 volts and small sting extensions rose and then dropped in the same manner as seen in 1 day olds. Large sting extension remains below the threshold at 2 volts but rises to about 70% at 4 volts. Small movements decrease as large movements and small stings increase but also decline as large sting extensions increase. The fixed effects comparison of the behavioural response levels with voltage increase shows a highly significant difference between the response changes as voltages increase ($F_{18,936} = 18.60$, $p = 0.0001$).

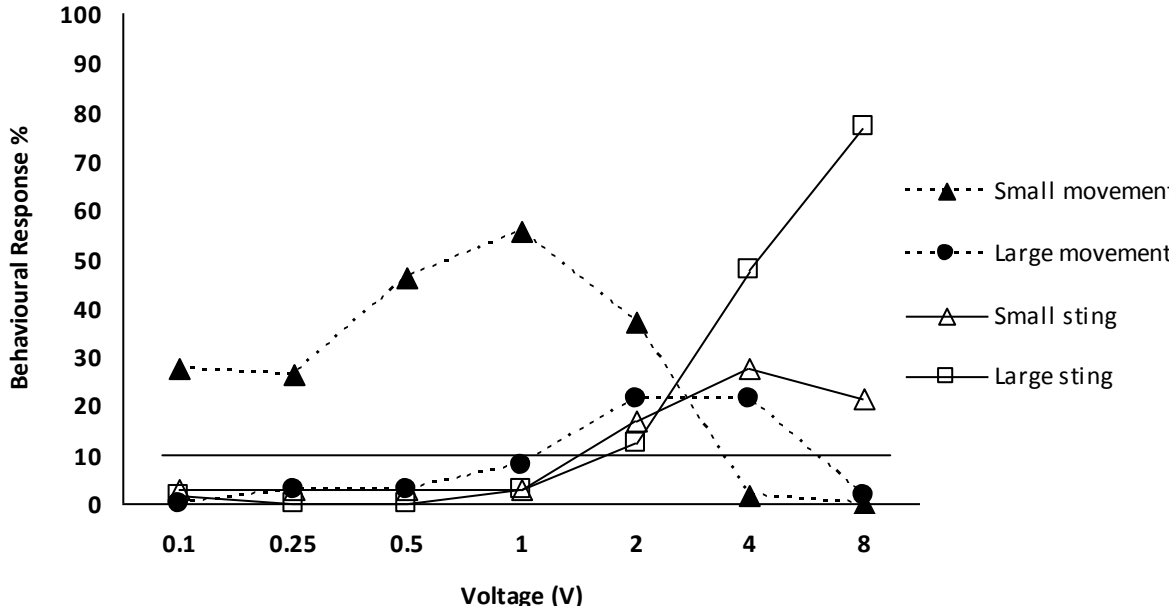

Figure 4.3. Defensive behavioural responses of 1 day old bees towards voltages from 0.1 to 8 volts. (n = 65).

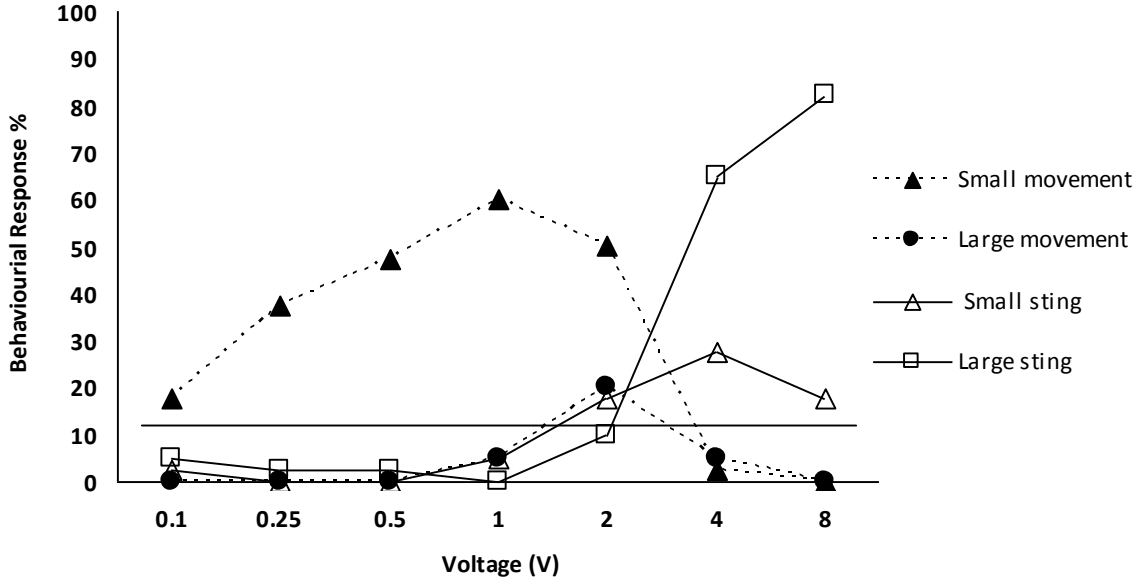

Figure 4.4. Defensive behavioural responses of 2 day old bees towards voltages from 0.1 to 8 volts. (n = 40).

In four day old bees the behavioural response rise of small movement was noticeably steeper and reached 77.5% by 0.5 volts (Fig. 4.5.). Its bell curve response was larger than other bees under one week of age. The large body movements did not rise above the threshold line (16.9%). Small sting extensions passed over the threshold at 2 volts and 4 volts but had declined at 8 volts. The large sting extension response remained well below the threshold until 4 volts and continued to increase at 8 volts. The fixed effects comparison of the behavioural response levels with voltage increase shows a highly significant difference between the response changes as voltages increase ($F_{18,936} = 21.80$, $p = 0.0001$).

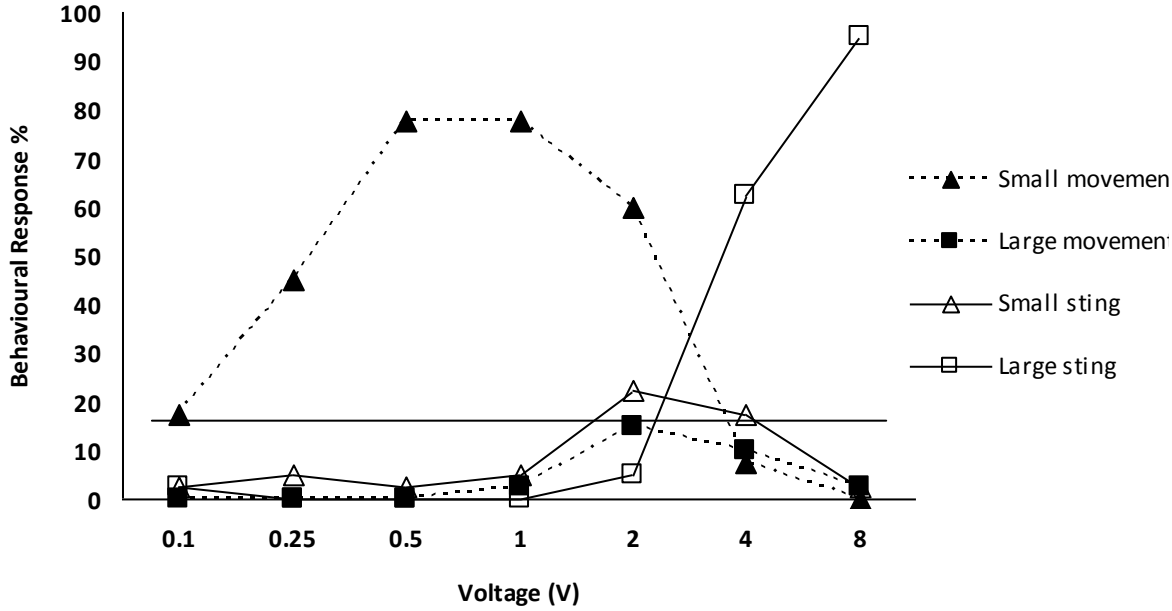

Figure 4.5. Defensive behavioural responses of 4 days old bees towards voltages from 0.1 to 8 volts. (n = 40).

A similar result seen in younger bees is repeated with 6 day old bees taken from the hive. Small movement is already above the threshold point (16.5%) at 0.1 volts (Fig. 4.6.). Large movement does not rise above the threshold line. Small and large sting extensions rose above the threshold line at 4 volts before small stings decreased at 8 volts while large stings continued to increase. The fixed effects comparison of the behavioural response levels with voltage increase shows a highly significant difference between the response changes as voltages increase ($F_{18,1392} = 28.44$, $p = 0.0001$).

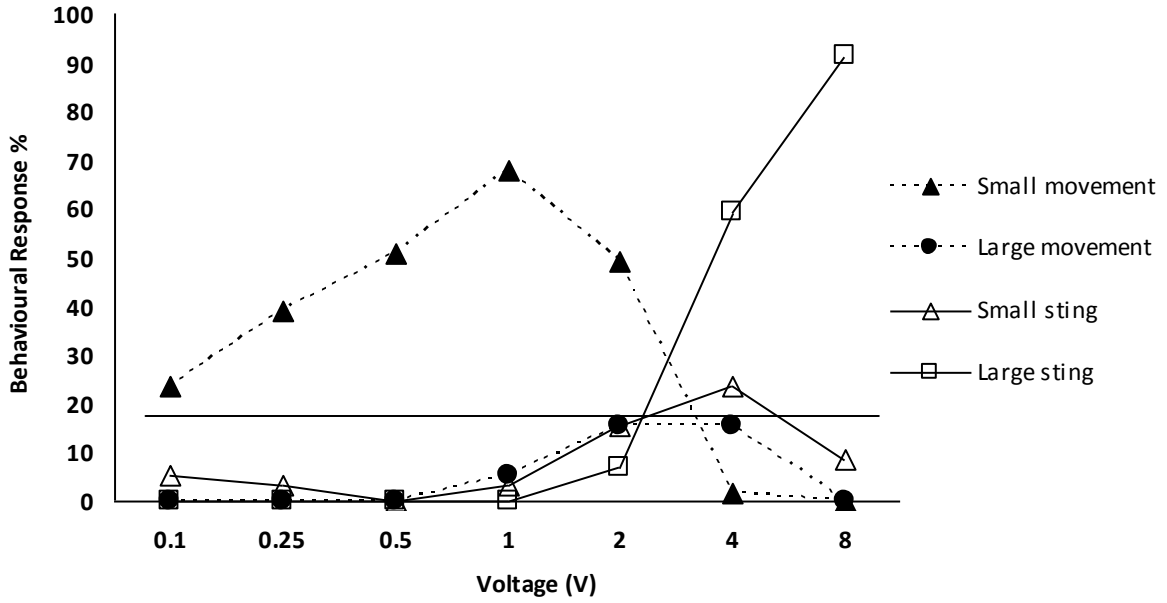

Figure 4.6. Defensive behavioural responses of 6 days old bees towards voltages from 0.1 to 8 volts. (n = 59).

Guard bees had two behavioural responses above the threshold (8.8%) at 0.1V (Fig. 4.7.). Their upper limit was calculated only from two behaviour responses because small sting extension response was unexpectedly higher than in previous groups. Small sting extension at 0.1 volts was at 15% and increased as small movements increased, but small stings dropped below the threshold at 2 volts only to rise above the threshold at 4 volts and fall below at 8 volts giving the behavioural group two peaks. However the two remaining behaviours did not register until 2 volts. Large sting extensions increased at similar rates seen in other groups tested. The fixed effects comparison of the behavioural response levels with voltage increase shows a highly significant difference between the response changes as voltages increase ($F_{18,936} = 21.09$, $p = 0.0001$).

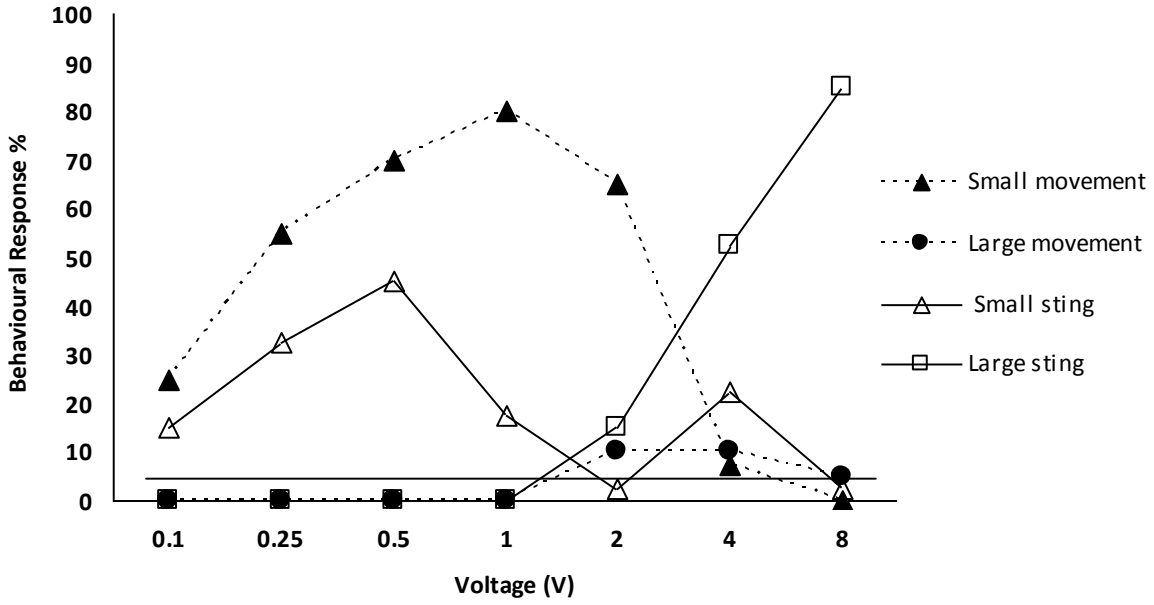

Figure 4.7. Defensive behavioural response of guard bees towards voltages from 0.1 to 8 volts. (n = 40).

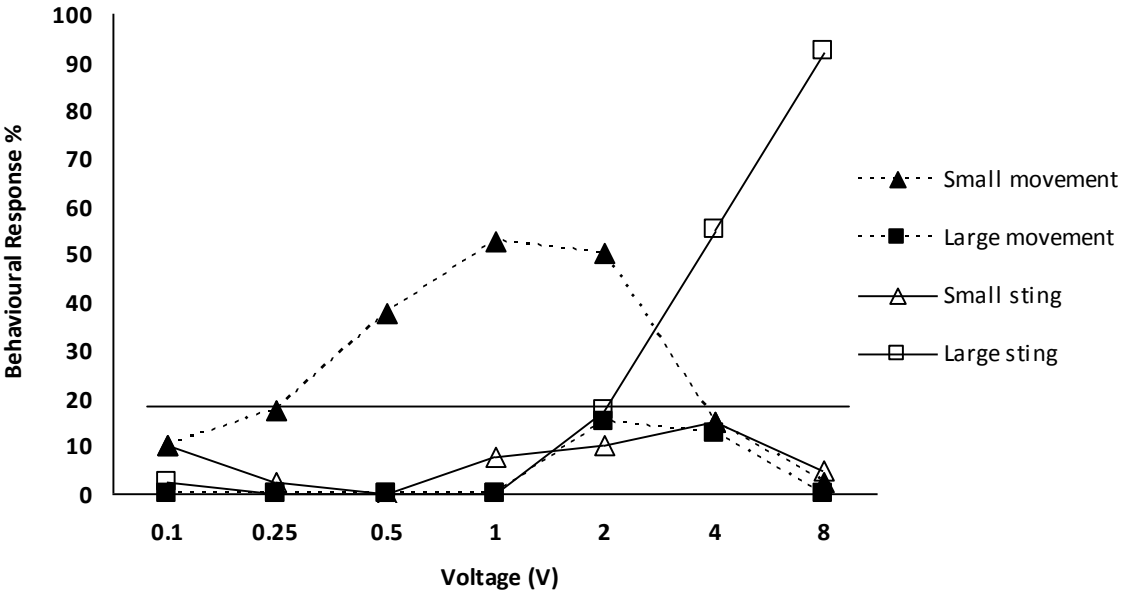

Figure 4.8. Defensive behavioural responses of pollen foragers towards voltages from 0.1 to 8 volts. (n = 40).

In pollen foragers all responses to 0.1 volts were below the threshold line of 18.6% (Fig. 4.8.). Starting at 10%, small movements rises to 37.5% at 0.5 volts, clear of the threshold and does not go below it until 4 volts. Large movement and small sting extension did not cross the threshold. Large sting extension passes the threshold at 4 volts and continues to rise at 8 volts. The fixed effects comparison of the behavioural response levels with voltage increase shows a highly significant difference between the response changes as voltages increase ($F_{18,936} = 14.88$, $p = 0.0001$).

Comparing small sting extensions from all groups gives a clear visual indication of the change in general response levels with increasing voltages (Fig. 4.9.). The response of small sting extensions from bees was significantly different ($\chi^2 = 88.594$, DF = 3, $p = 0.0001$). Response level of guard bees at 0.5 volts was significantly higher than all other groups (Sidak: 1&2 = 0.0001, 4&6 = 0.0001, pollen foragers = 0.0001).

Large sting extensions levels from the groups of bees were very close (Fig. 4.10.). The statistical comparisons of large sting extensions at various voltages were very similar among all age groups tested. A Chi-Squared test at 2 volts showed no statistical difference between groups ($\chi^2 = 3.85$, DF = 3, $p = 0.278$). However the same test showed a significant variation in the response levels at 8 volts between all groups ($\chi^2 = 9.879$, DF = 3, $p = 0.02$). Further testing using generalised linear model a pairwise comparison revealed the variation was occurring between 1&2 day olds and 4&6 day olds (Sidak $p = 0.02$).

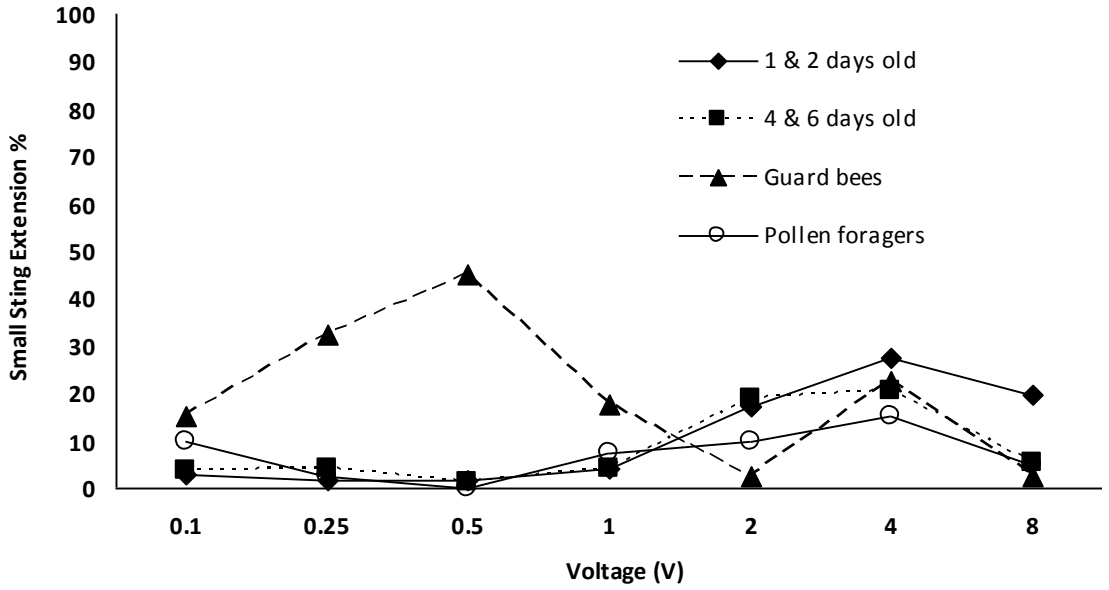

Figure 4.9. Small sting extensions from all age groups tested. An early response of SER among guard bees indicates they have a higher sensitivity response level than other groups tested.

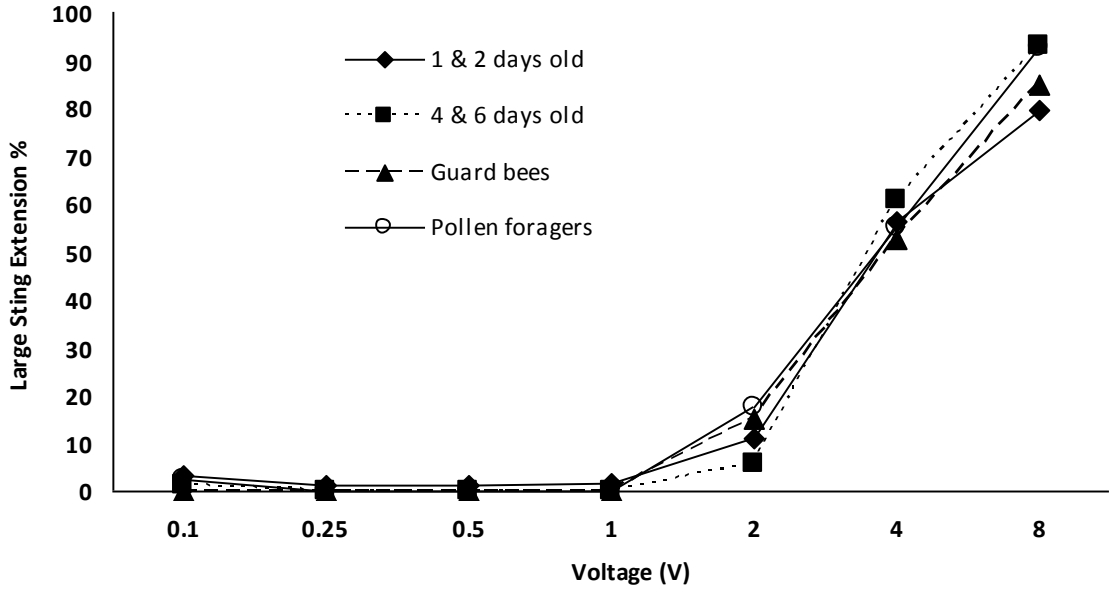

Figure 4.10. Large sting extensions from all age groups tested. A very similar response pattern between all bee groups throughout stimuli exposure.

4.2.2 Habituation to Aversive Stimuli

In contrast to the bees' responses to an odour puff, no group showed complete habituation to electric shock stimulation (Fig.4.11.). The exposure of 4 volts over 15 trials to young bees (1-6 day olds), guards and pollen foragers was not enough exposure trials for any of the groups to reach complete habituation. Guard bees habituated faster than other groups while bees less than one week old showed little habituation. The level of responses at trial 15, was significantly different across the four groups ($\chi^2 = 31.342$, DF = 3, p = 0.0001). The data were analysed again using a generalised linear model to determine where the differences lay. A pairwise comparison indicated that the groups of bees could be segregated into two groups (1 & 2 day olds and 4 & 6 day olds: (Sidak = 1) and Guards & Pollen foragers (Sidak = 0.276). Young bees (1 & 2 and 4 & 6 day olds) showed significantly less habituation than Guards & Pollen foragers (p = 0.0001).

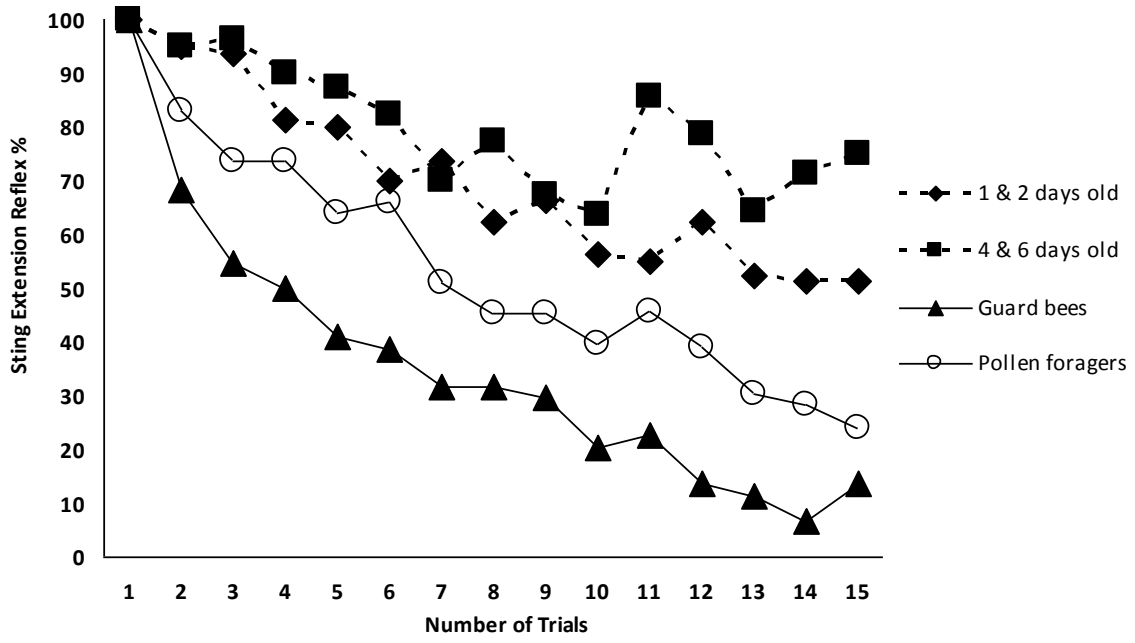

Figure 4.11. Habituation of the sting extension reflex in response to a mild electric shock stimulus (4 volts) delivered over 15 trials. (1 & 2 days n= 80; 4 & 6 days n= 60; Guard bees n= 44; Pollen foragers n= 53).

4.3 Aversive Learning

4.3.1 Development of Learning in Young Bees

4.3.1.1 Absolute Conditioning and 1 Hour Retention

Two day old bees collected during summer were raised in an incubator without QMP exposure to ensure the pheromone did not block possible learning (Vergoz et al., 2007b). The level of conditioned responses between trials 1-6 changed significantly (Cochran test, Q = 16.917, DF = 5, p = 0.005; Fig. 4.12.). A steep rise in the first three trials is likely to have contributed to the significant output. A small decrease of response levels was observed between trials 3-6. A comparative analysis between the sting extension reflex in trial one, prior to the electric shock, and the response level in the retention test one hour after the last conditioning trial showed no significant difference between the two (McNemar p = 0.344). This suggests bees that had learned to associate the odour with an aversive stimulus during the acquisition phase did not recall the memory 1 hour later.

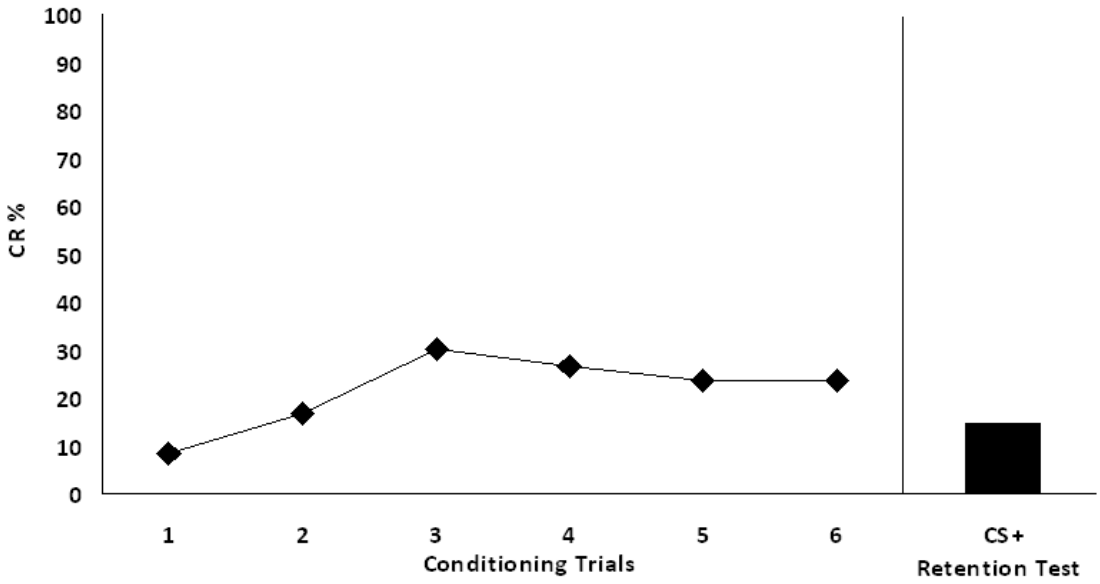

Figure 4.12. Two day old bees' learning response to absolute conditioning using eugenol paired with 7.5 volts and tested again one hour later for memory retention. (n = 60).

4.3.1.2 Differential Conditioning and 1 Hour Retention

During summer two and three day old bees were raised in an incubator without QMP exposure and were tested using a differential conditioning protocol. Differential conditioning of bees was followed 1 hour later with a retention test. CS+ (eugenol) was paired with a mild electric shock (7.5 volts). CS- (2-hexanol) was presented alone.

A significant change in response to CS+ occurred over the 6 conditioning trials of 2 day olds ($Q = 14.907$, $DF = 5$, $p = 0.011$; Fig 4.13.). A steep rise in response levels to the odour occurred in the first 3 trials similar to 2 day olds tested with absolute conditioning. It was observed that the response level in the CS+ 6th trial (11.6%) was below the response level recorded in the 1st trial (15%). Responses of two day old bees to CS- declined from 21.6% in the 1st trial down to 1.6% in the 4th trial ($Q = 27.77$, $DF = 5$, $p = 0.0001$). A comparison of responses between CS+ and CS- in the retention test showed no significant difference (McNemar: $\chi^2 = 13.067$, $DF = 1$, $p= 0.125$). Retention test responses to CS+ were only slightly higher than responses to CS-, expected of a group that could not retain memories. The comparison between the sting extension reflex in trial 1 and retention test response levels to CS+ was not significant (McNemar $p = 0.549$).

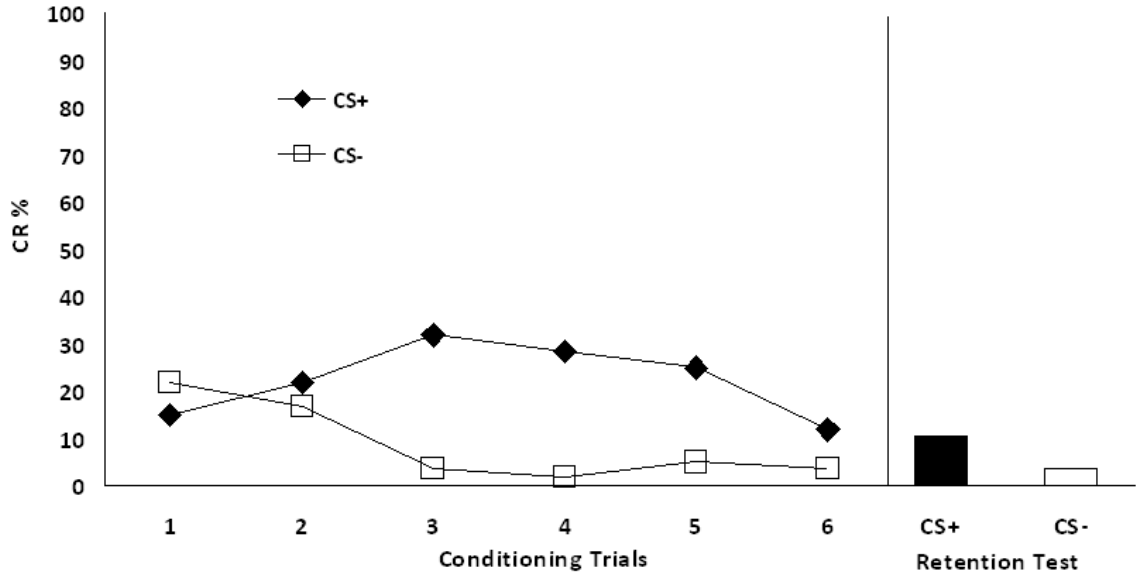

Figure 4.13. Percentage of two day old bees responding to an odour paired with a shock (CS+) and an odour that was not reinforced (CS-). A retention test was formed 1 hour after the last conditioning trial. (n= 60).

However a comparative analysis of sting extension reflex levels to CS- in trial 1 was significantly higher than the retention response levels (McNemar p = 0.001).

A comparison between absolute and differential conditioning in 2 day olds showed no difference between CS+ response levels in either retention tests (McNemar p = 0.549).

The number of 3 day old bees exhibiting sting responses to CS+ did not change significantly over the 6 conditioning trials (Q = 8.276, DF = 5, p = 0.142; Fig 4.14.). However the bees did demonstrate a change in responses to CS- that was highly significant (Q = 23.585, DF = 5, p <0.0001). Between trials 1-6, responses to CS- declined gradually. There was also a clear difference between CS+ and CS- response levels in the retention test with a greater number of sting extensions in response to CS+ odour than CS- (McNemar p = 0.0001). The percentage of three day olds responding to CS+ in the retention test was not statistically different to the percentage responding in trial 1 (McNemar p = 0.057). However fewer bees responded to CS- during the retention test than in the first conditioning trial (McNemar p = 0.001). The results show differentiation in 3 day olds if bees are not raised with QMP.

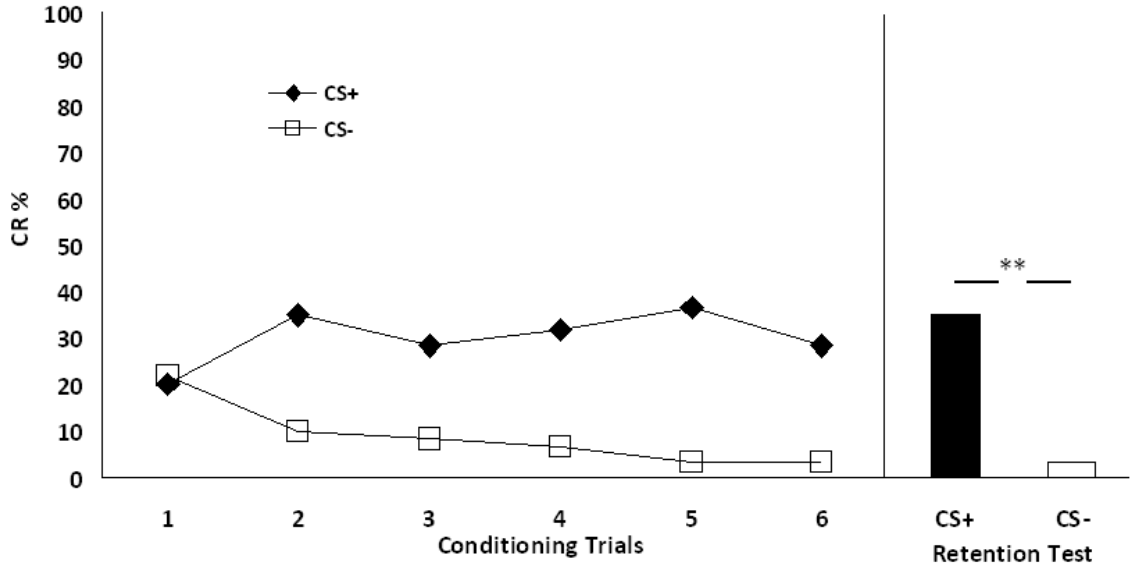

Figure 4.14. Percentage of three day old bees responding to an odour paired with a shock (CS+) and an odour that was not reinforced (CS-). A retention test was formed 1 hour after the last conditioning trial. (n= 60).

Three day old bees' response levels to a reinforced odour were significantly higher than 2 day olds during the retention test (McNemar p = 0.003). CS- response levels between 2 and 3 day olds showed no difference (McNemar = 1).

A Mann-Whitney test was used to compare the delta values (CS+ responses - CS- responses) of each conditioning between 2 and 3 day olds. Only the 5^{th} and 6^{th} trials in 3 days old bees had higher percentage of CS+ responses over CS- responses than in 2 day olds (Mann-Whitney test, 5^{th} trial: $Z_{adj} = 3.036$, $p = 0.002$; 6^{th} trial: $Z_{adj} = 2.739$, $p = 0.006$). In the retention test comparison there was a greater difference between CS+ and CS- response levels in 3 day olds than in 2 day olds ($Z_{adj} = 3.289$, $p = 0.001$).

4.3.2 Differential Conditioning and Retention in Older Bees

4.3.2.1 Learning in Summer Pollen Foragers and Guard Bees

Pollen foragers collected during October-January were considered summer pollen foragers. The percentage of pollen foragers responding to CS+ increased significantly over the 6 conditioning trials ($Q = 27.239$, $DF = 5$, $p < 0.0001$; Fig. 4.15.), whereas responses to CS- declined significantly over the conditioning trials ($Q = 13.659$, $DF = 5$, $p = 0.018$). In the retention test performed 24 hours after the last conditioning trial the bees responded to CS+ significantly more than to CS- (McNemar p = 0.0001). A comparison between response levels in the 1^{st} trial and response levels in the retention test showed a significant difference for CS+ (McNemar p = 0.011), but not for CS- (McNemar p = 0.508).

Guard bees tested were collected in the morning from the entrance of the hive during October-December. The graph shows the percentage of guard bees responding to CS+ and CS- during conditioning and during a retention test 24 hours after the last conditioning trial (Fig. 4.16). Guard bees' response levels to the reinforced odour (CS+) over the 6 conditioning trials increased significantly ($Q = 26.667$, $DF = 5$, $p = 0.001$), whereas no significant change was shown in their responses to the non reinforced odour (CS-) ($Q = 7.632$, $DF = 5$, $p = 0.178$). The responses to CS+ during the retention test were significantly higher than CS- (McNemar p = 0.0001), showing that the bees still discriminate between the

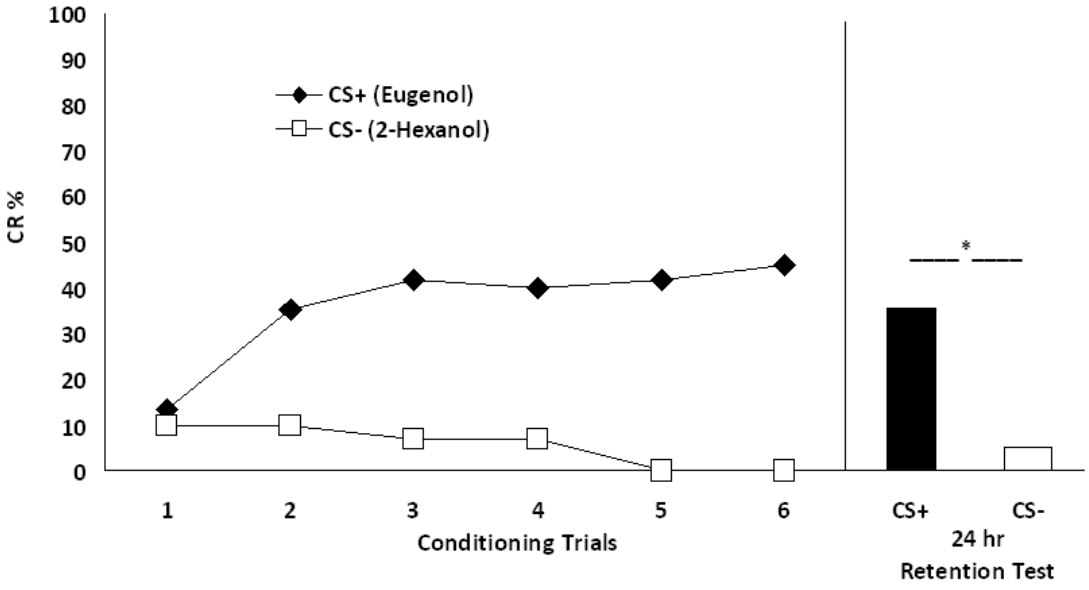

Figure 4.15. Pollen foragers collected during October-January from the hive entrance were tested using differential conditioning and then tested for memory retention 24 hours after the last conditioning trial. (n = 60).

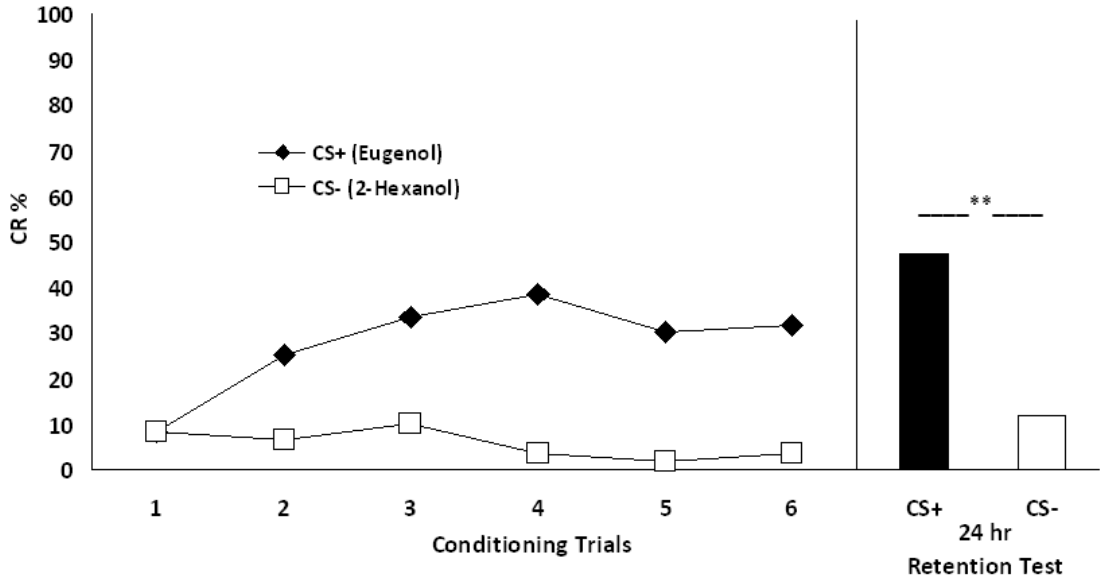

Figure 4.16. The guard bees were collected in October-December from the hive and were conditioned to associate one odour with an electric shock (CS+) and one odour with no reinforcement (CS-). The bees were then tested for retention 24 hours after the last conditioning trial. (n = 60).

two odours 24 hours after conditioning. The level of sting responses to CS+ recorded in the retention test was higher than in the first conditioning trial (McNemar p = 0.0005). However there was no difference in response levels to CS- retention test and in conditioning trial one (McNemar p = 0.754).

A McNemar test formed on the retention response levels between pollen foragers and guard bees 24 hours after the last conditioning trial showed no significant difference (McNemar: CS+ = 0.265, CS- = 0.344). The analysis indicated no difference in differentiating between odours and response levels in memory retention between the two labour groups. A Mann-Whitney analysis of summer pollen foragers and guard bees tested with differential conditioning and 24 hour memory retention, showed no significant difference between conditioning trials and retention response levels.

4.3.2.2 Memory Retention: 24 vs. 48 Hours

Pollen foragers collected from the hive entrance during summer months (December-February) were tested with differential conditioning using eugenol (CS+) and 2-hexanol (CS-) and tested 48 hours after the last conditioning trial (Fig. 4.17.). The percentage of bees responding to CS+ increased significantly over the 6 conditioning trials (Q = 22.217, DF = 5, $p < 0.001$). Responses to CS- also changed significantly (Q = 17.250, DF = 5, p = 0.004) with response levels dropping to 0% by trial 4. The reinforced odour (CS+) and non-reinforced odour (CS-) were both presented to the bees 48 hours after the last conditioning trial. Responses to CS+ and CS- in the retention test were different (McNemar: p = 0.016) with more bees responding to the CS+ odour. However there was no difference between the level of responses to CS+ in the 1st conditioning trial and in the 48 hour retention test (McNemar p = 1.00). This was true also for CS- (McNemar: p = 0.687).

Pollen foragers tested for retention of learnt memory and differentiation at 24 hours had a higher response level to CS+ than pollen foragers tested for memory retention at 48 hours (McNemar p = 0.043). While CS- response levels between the two groups were not different (McNemar = 1).

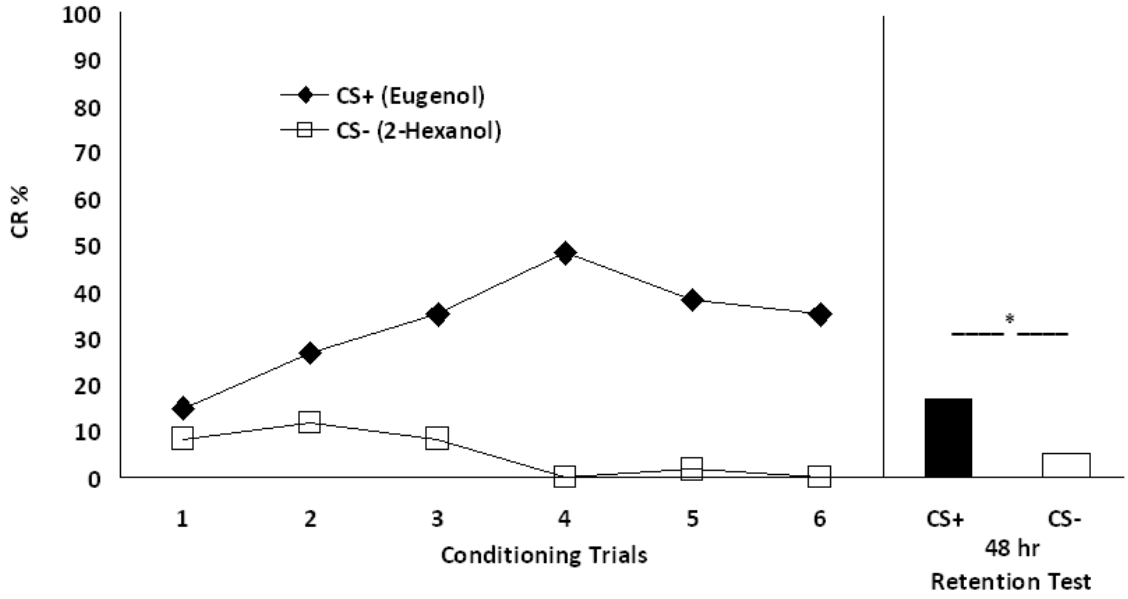

Figure 4.17. Pollen foragers collected during December-February from the hive entrance were tested using differential conditioning and then tested for memory retention 48 hours after the last conditioning trial. (n = 42).

A comparison of the conditioning trial delta values between summer pollen forager groups with 24 and 48 hours retention tests, using a Mann-Whitney test, was not significantly different at any trial. A Mann-Whitney test was used to compare the delta values of retention responses to CS+ and CS- odours from pollen foragers tested with 24 hours and 48 hours retention periods to find a significant difference ($Z_{adj} = 2.462$, $p = 0.014$). Pollen foragers tested at 24 hours after the final conditioning trial had higher response levels.

4.3.2.3 Seasonal Effects on Learning and Memory in Pollen Foragers

Pollen foragers collected at the hive entrance while returning back with pollen loads in August and September were considered as winter pollen foragers. The response levels to CS+ declined slightly over the last 4 conditioning trials while CS- responses declined greatly after the 2nd trial. A Cochran test showed there was no significant change in the number of bees responding to CS+ over the 6 conditioning trials ($Q = 6.011$, $DF = 5$, $p = 0.305$; Fig 4.18.). However a highly significant change in responses to CS- occurred as a result of response conditioning ($Q = 41.217$, $DF = 5$, $p < 0.0001$).

In the retention test formed 24 hours after the last conditioning trial the response level to the reinforced odour (CS+) was higher than the level of responses to the non reinforced odour (CS-) (McNemar p= 0.001). A comparative test for each odour between response levels in the first trial and the level of responses recorded during the retention test showed a significant difference for both odours, CS+ (McNemar p= 0.017) and CS- (McNemar p= 0.004).

A comparison of retention response levels to both odours between winter and summer pollen foragers showed no significant difference (McNemar: CS+ = 0.176, CS- = 1). The level of retaining memories is the same in both pollen forager groups despite seasonal differences seen during conditioning trials. A Mann-Whitney test between summer and winter pollen foragers found two delta response levels during conditioning trials to be significantly different (2^{nd} trial $Z_{adj} = 2.847$, $p = 0.004$; 6^{th} trial $Z_{adj} = 1.961$, $p = 0.050$). Another comparison between the two delta retention test values was non-significant ($Z_{adj} = 1.003$, $p = 0.316$).

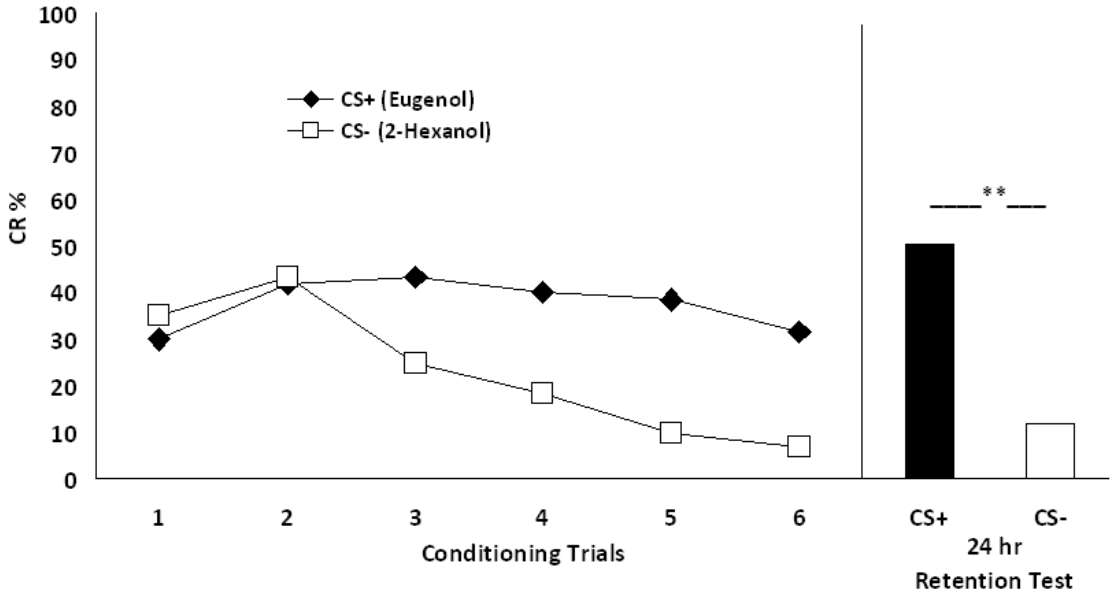

Figure 4.18. Pollen foragers collected in August-September from the hive were tested using differential conditioning and then tested for memory retention 24 hours after the last conditioning trial. (n = 60).

4.4 Extinction of Aversive Memories

Only bees that showed a conditioned response in the retention test were used in this experiment. The extinction rate was formed by the response levels to CS+ odour after the retention test (Fig. 4.19.). The declining responses R^2 values were calculated for guards (R^2 = 0.972), winter (R^2= 0.997) and summer foragers (R^2 = 0.943). The graphs shows guard bees reaching a nonresponsive state first. A two sample t-test indicated guards' sting extension reflex regression was significantly different from winter pollen foragers (T-value = 13.96, DF = 15, p = 0.0001) and summer pollen foragers (T-value = 8.43, DF = 27, p = 0.0001).

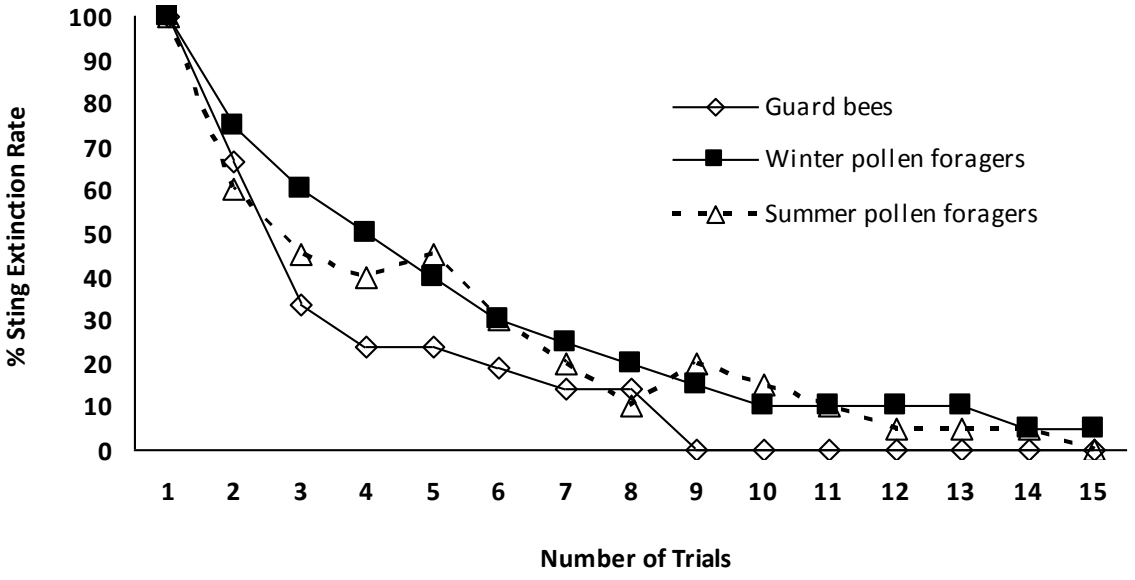

Figure 4.19. Extinction rate of sting responses towards CS+ odour following the 24 hour retention test. Determining if there is a labour related difference in forming new response association over the preconditioned ones. (Guard bees n= 21; Winter pollen foragers n= 20; Summer pollen foragers n= 20).

4.5 Changes in Response to CS-

The declining response to CS- of bees has been taken from differential conditioning trials and included two groups of pollen foragers exposed to the same odour (2-hexanol) alone at different interval lengths, 10 and 20 minutes. This will enable the comparison of the response decline between age and labour groups (Fig. 4.20.), and the effect of inter stimulus intervals on response decline (Fig. 4.21).

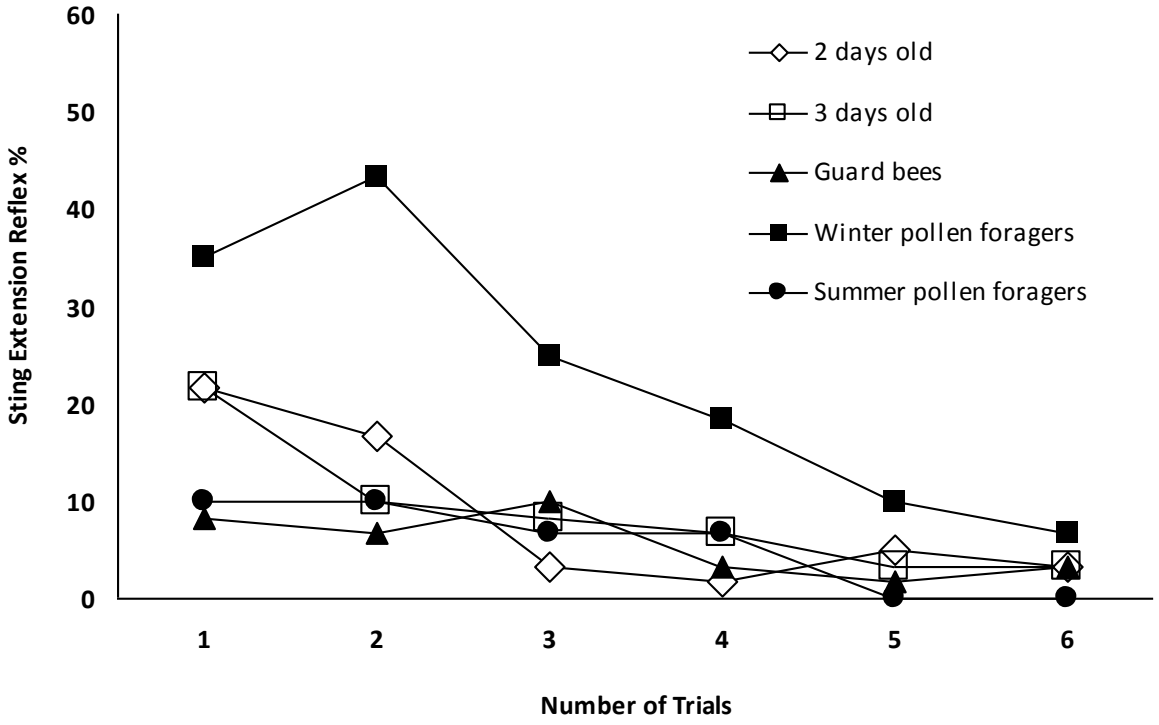

Figure 4.20. Reduction of sting extension response to CS- from all age groups tested in differential conditioning. (2 day olds n= 60; 3 day olds n= 60; Guard bees n= 60; Winter pollen foragers n= 59; Summer pollen foragers n= 60).

A linear regression of 2 and 3 day olds showed a similar rate of decline (slope; 2 days: -3.667 and 3 days: -3.238. Appendix A: Table 1). An univariate ANOVA showed no significant difference between the two slopes, suggesting a similar decreasing rate of response levels to CS- in young bees ($F_{1,8} = 0.078$, $p = 0.787$).

The guard bees' responses to CS- had the lowest rate of decline among the three older age groups (slope = -1.333. Appendix A, Table 2). Winter pollen foragers have the largest decline rate (slope = -7.095) and largest standard error (S.E = 1.404) because this group had a high initial response rate (35%, Figure 4.6.1). Summer pollen foragers' response decline was significantly different to a line of no change (p = 0.005). Univariate ANOVA comparison between guards and winter pollen foragers showed there was a gradient difference of 5.762, greatly influenced by winter bees' high sting response in trial 1 and 2 ($F_{1,8}$ = 14.472, p = 0.005). Gradient response levels of guard bees and summer pollen foragers are very similar, 0.952 and when analysed no difference was found ($F_{1,8}$ = 1.821, p = 0.214). A comparison between winter and summer pollen foragers revealed a difference in the decline of CS- response levels between the two groups ($F_{1,8}$ = 10.776, p = 0.011).

The gradient slope difference between pollen foragers with 10 and 20 minute intervals were very similar (Appendix A, Table 3). An ANOVA univariate tested the decline in response levels of summer pollen foragers (CS-) and pollen foragers with inter stimulus intervals of 10 and 20 minutes. The tests revealed summer pollen foragers with a mixture of 10 and 20 minute inter stimulus intervals was significantly different to pollen foragers tested with 20 minute inter stimulus intervals ($F_{1,8}$ = 5.483, p = 0.047). The comparisons between response levels of summer pollen foragers (CS-) and pollen foragers tested with 10 minute inter stimulus intervals ($F_{1,8}$ = 1.651, p = 0.235) and pollen foragers tested with 20 minute inter stimulus interval versus 10 minutes ($F_{1,8}$ = 0.334, p = 0.579) were not different.

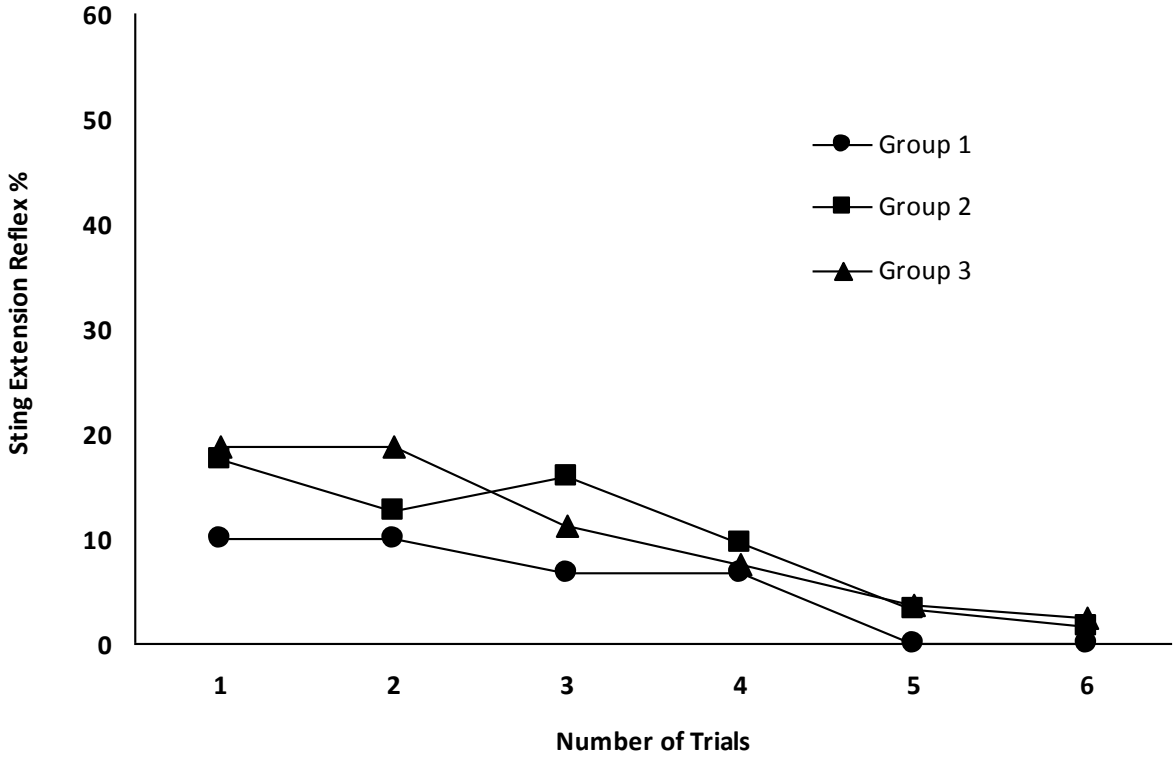

Figure 4.21. Comparison of the decline in responses to an odour used as CS- in differential conditioning paradigm and an odour used to test habituation. (Group 1: Summer pollen foragers' CS- responses with a mixture of 10 & 20 minute inter stimulus intervals n= 60; Group 2: Pollen foragers with 10 minute inter stimulus interval, n= 63; Group 3: Pollen foragers with 20 minute inter stimulus intervals, n= 80).

5 Discussion

5.1 Age-related Changes in Responsiveness to Non-Threatening Stimuli

This study shows bees demonstrate interesting labour and age-related changes in responsiveness to sensory stimuli. The sting extension reflex is a defensive response that can be used to study response thresholds to stimuli. During the first 4 days of adult life the bees responded strongly to novel stimuli (Fig.4.1). Defensive responses to air and odour puffs peaked in 4 day olds suggesting that bees of this age are particularly sensitive to tactile stimuli. However older bees (6 day olds, guards and pollen foragers) appeared to be less responsive to novel tactile stimuli, especially air puffs.

This change in responsiveness between 4 and 6 day olds might be under hormonal control. Juvenile hormone levels, for example, begin to rise at 4 days of age (Velarde et al., 2009). The hormone rise may affect the bees' sensitivity to antennal stimulation in preparation for foraging (Jaycox, 1976; Robinson, 1987; Huang and Robinson, 1999). Juvenile hormone plays an important role in age polyethism, influencing the movement from specialised hive tasks to foraging and response changes to colony pheromones (Robinson, 1992). Hormonal control was demonstrated with young bees performing tasks precociously after treatment with the juvenile hormone analog methoprene (Robinson, 1987). In the mushroom bodies of the brain, juvenile hormone triggers an internal reorganisation of neural circuits which may be responsible for changing the bee's sensitivity to sensory stimuli (Withers et al., 1995; Fahrbach and Robinson, 1996). Neural plasticity in the brain enables older bees to cope with the demanding task of foraging (Robinson, 1987; Fahrbach and Robinson, 1996).

Biogenic amines are also involved in the control of division of labour and may modulate olfactory response thresholds to task related stimuli (Beshers et al., 1999; Schulz and Robinson, 1999). Biogenic amine levels in the mushroom bodies and in the antennal lobes increase with age (Taylor et al., 1992; Durst et al., 1994; Harris and Woodring, 1995; Schulz and Robinson, 1999; Wagener-Hulme et al., 1999) and amine level changes have been found to correlate with the performance of different behavioural tasks (Wagener-Hulme et al., 1999). Octopamine levels in the brain, for example, are low in nurse bees but increase

with age and are associated with foraging (Taylor et al., 1992; Wagener-Hulme et al., 1999). Increasing octopamine levels experimentally in 4 day old nurses induces the onset of precocious foraging (Schulz and Robinson, 1999). In moths, octopamine has been shown to modulate activity at the peripheral level (Pophof, 2000; Brigaud and Grosmaitre, 2008). An increase in octopamine levels in the moth also increases the activation in olfactory receptor neurons, strongly influencing response thresholds (Pophof, 2000; Grosmaitre et al., 2001).

Interestingly, 6 day olds and pollen foragers responded with sting extension more to odour puffs than air puffs perhaps because of the stronger saliency of an odour puff. Such differences in responses to air and odour puffs were not seen in young (1-4 day old) bees or in guards. Responses to puffs of air or odour were particularly low in the guard bees. The low response levels to odour stimuli from guards may be related to their role in policing the entrance of the hive. Guard bees on duty are reported to be more responsive to intruders than they are to floral odours (Bowden et al., 1998). Guards recognise honey bee intruders by their distinctive colony odour (Moritz and Hillesheim, 1990). They are also more responsive to alarm pheromone than other behavioural groups (Moore et al., 1987b; Breed et al., 1989). A higher sting extension threshold for tactile and olfactory stimuli in guards may be advantageous for a guard bee. It is possible that guards avoid using excessive energy by not responding to every stimulus they encounter and only responding to noxious stimuli that threaten the colony. Guarding behaviour and sting extension are influenced by genetic traits (Guzmán-Novoa et al., 2002; Arechavaleta-Velasco et al., 2003; Lenoir et al., 2006; Uribe-Rubio and Guzman-Novoa, 2008). Bees that become guards are found to have a higher frequency of an allele marker that is located next to a quantitative trait known to be a phenotypic characteristic for stinging behaviour (Hunt et al., 1998; Guzmán-Novoa et al., 2002; Arechavaleta-Velasco et al., 2003).

All bees that responded to the odour puff also habituated to repetitive odour puffs. Initial response levels to an odour puff among 1-6 day olds and pollen foragers were not significantly different (Fig. 4.1). However 1 & 2 day olds habituated very rapidly to repetitive odour puffs, faster than 4 & 6 day olds and pollen foragers (Fig. 4.2). These results suggest that the nervous system of younger worker bees is possibly still undergoing developmental changes.

There is evidence suggesting that amine levels influence response habituation. Depletion of amine levels in the nervous system of worker bees is reported to lead to a 30% decline in the number of bees displaying proboscis extension reflex in response to sucrose stimulation of the antennae (Braun and Bicker, 1992). Braun and Bicker found that the proboscis extension of the remaining responsive bees habituated more rapidly to the repetitive stimulation with sucrose than the control group. Treatment with octopamine was found to restore the proboscis reflex in unresponsive bees (Braun and Bicker, 1992).

Further investigation into habituating responses could include examining response levels to the habituated odour 24 hours following the 15th trial. A low response level may demonstrate bees had learnt and remembered the odour is not a threat. Another test may include a second odour on the 16th exposure trial to be exposure to the bees. Introducing a new odour may indicate if bees are habituating to the specific odour fragrance or to repetitive tactile stimulation.

5.2 Responses to Aversive Stimuli

All bee groups examined in this study responded to aversive stimuli. At the lowest voltages used (0.1-1 volts) small body movements were observed in some bees. A stimulation of 1 volt induced large body movement and, in some bees, small sting extensions were seen until finally, at the highest voltages used, large sting extensions were induced. Most bees showed little or no response to 0.1 volt stimuli, but some 1- 6 day olds showed small body movements. Interestingly, some guard bees also responded to 0.1 volts, not only with small body movements but also with small sting extensions. Guard bees were more sensitive to the aversive stimuli than other bee groups, partially extending their sting in response to the lowest voltages presented. This can be clearly seen in Fig. 4.9. For most bee groups tested the threshold for small sting extensions was between 0.5 and 2 volts but in guards the threshold appears to be lower than the 0.1 volts used. Guards have been reported elsewhere to be less sensitive to aversive stimuli than foragers (Roussel et al., 2009). However the study by Roussel et al. (2009) used a range of stimuli from 0.25-8 volts with only foragers and they recorded large sting extensions only, smaller responses were not

reported. The study suggested aversive responses of sting extension increases as the strength of the aversive stimuli increases.

Sensitivity to negative stimuli has a significant genetic component and varies within a colony depending on the patriline (Guzmán-Novoa and Page, 1994; Guzmán-Novoa et al., 2002; Lenoir et al., 2006; Uribe-Rubio and Guzman-Novoa, 2008). However guard bees are generally more aggressive towards threats than any other members of the colony (Breed et al., 1990; Breed et al., 1992). The results of this study suggest that a small aggravation to guard bees will stimulate a proportion of this group to respond with sting extension while the same aggravation to other groups produces only small body movement.

The threshold for large sting extensions was very similar in all groups tested and ranged between 1 and 2 volts. Changes in age or behaviour seem not to significantly affect the large sting extension response to increasing voltages. The results indicate that large sting extension is the main behavioural response to strong negative stimuli. Although the response threshold for large sting extension was similar in all groups, repeated stimulation using a 4 volt stimulus revealed significant differences in habituation rate between young and old worker bees (Fig. 4.11). Bees less than one week old habituated more slowly than older bees. Differences in the rate of sting extension decline is further evidence that sensory information processing in younger bees differs from that in older bees. However it is not currently known why guards are more sensitive to low level voltages than other groups and habituate more quickly to a mild aversive stimulus than other groups.

5.3 Aversive Learning and Memory Retention

This study has shown 2 and 3 day old bees are not able to retain aversive associations. However, they do appear to learn which odour is not a threat and retain this memory for at least 1 hour. Two and 3 day old bees showed poor aversive learning and no evidence of aversive memory recall one hour after the last conditioning trial. The conditioned response levels in the retention test did not differ significantly from the level of responsiveness to the CS+ odour in the very first conditioning trial before reinforcement was given. While in the 2 day olds examined, the percentage of bees responding to CS+ increased over the first 3 conditioning trials, this was not apparent in the 3 day olds. Also, the response levels to CS+

in absolute and differential conditioning of 2 day olds declined from conditioning trials 4 to 6. A decline in the percentage of bees displaying a conditioned response in trials 4 to 6 was also observed in a study by Behrends and Scheiner (2009) who examined appetitive learning in one day old bees. Their study showed an increasing proboscis extension response from trial 1 to 3, but a decline in conditioning trials 4-6. The poor appetitive olfactory learning in young bees compared to foragers was attributed to only 22% of young bees being able to respond to sucrose stimulation with a proboscis extension. The study showed olfactory learning could be demonstrated in only responsive young bees. Young bees have been shown to be less responsive to sucrose stimulation than older bees (Morgan et al., 1998; Pankiw and Page, 1999), affects their ability to form olfactory associations (Scheiner et al., 2001).

Although young bees did not show strong aversive learning, they did show consistent changes in responsiveness to CS-. A decline in responses to CS+ from conditioning trials 1 to 6 was shown in both young bee groups suggesting that young bees do learn which odour not to respond to. Young bees also appeared to retain information about which odour not to respond to more readily than learning that a particular odour is associated with a punishment. The ability of 3 day olds to differentiate between CS+ and CS- in the retention test also appears to depend on their ability to recall that CS- offers no threat, rather than any memory of an association between CS+ and punishment. Evidence in the fruit fly, *Drosophila*, indicates that the formation of aversive memories occurs in the mushroom bodies and relies on the neuromodulator dopamine (Schwaerzel et al., 2003). Dopamine levels in the mushroom bodies of young bees are lower than foragers (Schulz and Robinson, 1999), which may help explain why aversive learning is better in older bees than in younger bees. However, the declined rate of sting extension responses to CS- was not affected by age, behaviour or the presence of a reinforced odour (Fig. 4.20; 4.21).

Levels of response to CS+ and CS- observed in pollen foragers in the present study are similar to those described in previous studies (Vergoz et al., 2007a; Roussel et al., 2009). In contrast to the present investigation, Roussel et.al (2009) found that guard bees were less responsive to aversive stimuli than the forager group they used in their study, and showed poorer learning. In the present study, responsiveness to negative stimuli and aversive learning levels in guards were similar to the response levels observed in summer pollen foragers. However, sting extension responses to CS+, indicating retained aversive memories, were

extinguished more rapidly in guards than pollen foragers (Fig. 4.5). The absence of reinforcement meant that the odour quickly lost its influence over the defensive reflexes within 15 trials. The rapid decline in responses observed in guard bees may be related to the low level responses guards show to novel air and odour puffs.

The behaviour of guards described in the present study is however consistent with the response threshold model described by Roussel et al. (2009). They found bees with a lower threshold, responding more to a negative stimulus, show better learning and consequently retain memories related to the conditioned stimulus better than individuals with a higher stimulus response threshold.

5.4 Memory Retention: 24 vs. 48 Hours

All previous studies of aversive learning in honey bees have examined memory retention 1 hour after the last conditioning trial. This study shows that pollen foragers are able to retain aversive memories for 24-48 hours. Over 40% of the pollen foragers collected in summer learned to associate an aversive stimulus with punishment and in the retention test after 24 hours 35% could still differentiate between the two odours. In some pollen foragers aversive memories are retained for at least 48 hours after the final conditioning trial (Fig. 4.17). However the level of conditioned responses is significantly lower after 48 hours than 24 hours. After 48 hours 16% of pollen foragers still have the ability to differentiate between the two odours, but this appears to depend on bees showing a low response to CS- rather than an enhanced response to CS+. After 48 hours, responses to CS+ were not significantly higher than the spontaneous response levels observed in the initial exposure to the CS+ odour.

The results from this study, in comparison to previous studies of appetitive retention, suggest that aversive memories do not last as long as appetitive memories. The literature shows that after 3 conditioning trials, appetitive memories last at least 2 weeks (von Frisch, 1967; Menzel, 1968; Menzel and Erber, 1978) without indication of response decline (Menzel and Erber, 1978; Gerber et al., 1998; Wüstenberg et al., 1998; Menzel, 1999). However the retention duration is dependent on the strength of the stimulus (Menzel, 1968; Bitterman et al., 1983), the amount of time between conditioning trials and the length of stimulus exposure (Bitterman et al., 1983; Gerber et al., 1998). It has also been observed that

conditioned response levels following aversive conditioning are generally lower than after appetitive conditioning (Unoki et al., 2005; Vergoz et al., 2007a; Vergoz et al., 2007b), as a result formation of aversive olfactory associations and memory are lower. It is possibly advantageous for a bee to learn aversive associations quickly, but just as important to have these memories decline after 1-2 days. An aversive stimulus in the field, a spider attack on a specific flower, for example, may not be present later that day (Dukas, 2001; Dukas and Morse, 2003). Continuous avoidance of the food source location may become a disadvantage as the event would be one off or no longer in the same location. Further investigation of the retention of aversive olfactory memories could examine the length of time responses to CS+ remain.

5.5 Seasonal Effects on Learning and Memory in Pollen Foragers

Interestingly aversive olfactory learning in pollen foragers appears to be affected by season (Fig 4.15, Fig 4.18). Winter bees showed no significant change in levels of responsiveness to CS+ over the 6 conditioning trials. But retention tests showed that winter pollen foragers do form and retain aversive olfactory memories. No significant change in responses during aversive olfactory learning in winter bees might be related to the high level of 'spontaneous' responses to CS+ odour, particularly in the initial response of the first conditioning trial. Initial responses to CS- were also high, however winter pollen foragers showed a strong response decline to CS-. Winter pollen foragers also maintained low level responses to CS- during the 24 hour retention test.

During winter, bees become generalists, performing tasks within the hive, similar to nurse bees (Johnson, 2010). However they may adventure outside to forage, weather permitting (Sekiguchi and Sakagami, 1966). During winter the absence of brood pheromone within the hive increases the level of vitellogenin in the adipose tissue and in the hemolymph of workers as they age (Amdam et al., 2004; Smedal et al., 2009). The increase of vitellogenin in the hemolymph prolongs the life span of winter bees beyond the life span observed in summer bees (Maurizio, 1950; Fluri et al., 1982; Omholt, 1988). The change of vitellogenin levels between winter and summer months may be responsible for affecting the

responsiveness of bees to sensory stimuli. This could explain the unusual acquisition curves observed in winter bees.

Overall this thesis has identified age and caste related differences in responsiveness to novel air and odour stimuli, with guard bees being least responsive. However, guards were more sensitive to low level voltages and habituated to repetitive exposures of 4 volts more rapidly than other groups. Aversive olfactory learning in 2 and 3 day olds was poor and there was no memory retention for the conditioned stimulus (CS+) 1 hour after conditioning. However, young bees did show associative olfactory learning and olfactory memory retention to the CS- odour. Pollen foragers demonstrated that aversive olfactory memories could be retained for 48 hours, but responses begin to diminish after 24 hours. Aversive olfactory learning and memory in guards was similar to summer pollen foragers but not in winter pollen foragers. Lastly, extinction of aversive olfactory memories had declined more rapidly in guard bees than foragers.

6 References

Amdam GV, Omholt SW. 2003. The hive bee to forager transition in honeybee colonies: The double repressor hypothesis. *Journal of Theoretical Biology* **223**:451-464.

Amdam GV, Hartfelder K, Norberg K, Hagen A, Omholt SW. 2004. Altered physiology in worker honey bees (Hymenoptera: Apidae) infested with the *Varroa destructor* (Acari: Varroidae): A factor in colony loss during overwintering? *Journal of Economic Entomology* **97**:741-747.

Amdam GV, Omholt SW. 2003. The hive bee to forager transition in honeybee colonies: The double repressor hypothesis. *Journal of Theoretical Biology* **223**:451-464.

Arechavaleta-Velasco ME, Hunt GJ, Emore C. 2003. Quantitative trait loci that influenced the expression of guarding and stinging behaviours of individual honey bees. *Behavior Genetics* **33**:357-364.

Balderrama N, Núñez J, Guerrieri F, Giurfa M. 2002. Different functions of two alarm substances in the honeybee. *Journal of Comparative Physiology A: Neuroethology, Sensory, Neural, and Behavioral Physiology* **188**:485-491.

Beggs KT, Glendining KA, Marechal NM, Vergoz VN, I., Slessor KN, Mercer AR. 2007. Queen pheromone modulates brain dopamine functions in worker honey bees. *Proceedings of the National Academy of Sciences of the United States of America.* **104**:2460-2464.

Behrends A, Scheiner R. 2009. Evidence for associative learning in newly emerged honey bees (*Apis mellifera*). *Animal Cognition* **12**:249-255.

Beshers SL, Robinson GE, Mittenthal JE. 1999. The response threshold concept and division of labour. *Information Processing in Social Insects*. Birkhäuser Verlag, Basel. 115-141.

Bevan E. 1843. *The Honey Bee; Natural History, Physiology, and Management*. Carey and Hart, Philadelphia.

Bitterman ME, Menzel R, Fietz A, Schäfer S. 1983. Classical conditioning of proboscis extension in honeybees (*Apis mellifra*). *Journal of Comparative Psychology* **97**:107-119.

Boch R, Morse RA. 1974. Discrimination of familiar and foreign queens by honey bee swarms. *Annals of the Entomological Society of America* **67**:709-711.

Bowden RM, Williamson S, Breed MD. 1998. Floral oils: Their effect on nestmate recognition in the honey bee, *Apis mellifera*. *Insectes Sociaux* **45**:209-214.

Braun G, Bicker G. 1992. Habituation of an appetitive reflex in the honeybee. *Journal of Neurophysiology* **67**:588-598.

Breed MD. 1981. Individual recognition and learning of queen odors by worker honeybees. *Proceedings of the National Academy of Sciences of the United States of America* **78**:2635-2637.

Breed MD. 1983. Nestmate recognition in honey bees. *Animal Behaviour* **31**:86-91.

Breed MD, Guzmán-Novoa E, Hunt GJ. 2004. Defensive behavior of honey bees: Organization, genetics, and comparisons with other bees. *Annual Review of Entomology* **39**:271-298.

Breed MD, Robinson GE, Page RE. 1990. Division of labor during honey bee colony defense. *Behavioral Ecology and Sociobiology* **27**:395-401.

Breed MD, Rogers KB. 1991. The behavioral genetics of colony defense in honeybee: Genetic variability for guarding behavior. *Behavior Genetics* **21**:295-303.

Breed MD, Rogers KB, Hunley JA, Moore AJ. 1989. A correlation between guard behavior and defensive response in the honeybee, *Apis mellifera*. *Animal Behaviour* **37**:515-516.

Breed MD, Smith TA, Armando T. 1992. Role of guard bees (*Hymenoptera: Apidae*) in nestmate discrimination and replacement of removed guards. *Entomological Society of America* **85**:633-637.

Brigaud I, Grosmaitre X. 2008. Cloning and expression pattern of a putative octopamine/tryamine receptor in antennae of the noctuid moth *Mamestra brassicae*. *Cell Tissue Research* **335**:455-463.

Bruce TJ, Cork A, Hall DR, Dunkelblum E. 2002. Laboratory and field evaluation of floral odours from African marigold, *Tagetes erecta*, and sweet pea, *Lathyrus odoratus*, as kairomones for the cotton bollworm *Helicoverpa armigera*. *International Organisation for Biological and Integrated Control of Noxious Animals and Plants, WPRS, Bulletin* **25**:1-9.

Cartwright BA, Collett TS. 1983. Landmark learning in bees. *Journal of Comparative Physiology A: Neuroethology, Sensory, Neural, and Behavioral Physiology* **151**:521-543.

Cobb M. 2001. Reading and writing The Book of Nature: Jan Swammerdan (1637-1680). *Endeavour* **24**:122-128.

Collins AM. 1980. Effect of age on the response to alarm pheromones by caged honeybees. *Annals of the Entomological Society of America* **73**:307-309.

Craig CL. 1994. Predator foraging behavior in response to perception and learning by its prey: interactions between orb-spinning spiders and stingless bees. *Behavioral Ecology and Sociobiology* **35**:45-52.

Craig CL, Ebert K. 1994. Colour and pattern in predator-prey interactions: The bright body colours and patterns of a tropical orb-spinning spider attract flower-seeking prey. *Functional Ecology* **8**:616-620.

Crane E. 1983. *The Archaeology of Beekeeping*. Duckworth, London.

Deisig N, Giurfa M, Sandoz J. 2010. Antennal lobe processing increases separability of odor mixture representations in the honeybee. *Journal of Neurophysiology* **103**:2185-2194.

Dukas R. 2001. Effects of perceived danger on flower choice by bees. *Ecology Letters* **4**:327-333.

Dukas R, Morse DH. 2003. Crab spiders affect flower visitation by bees. *Oikos* **101**:157-163.

Durst C, Eichmüller S, Menzel R. 1994. Development and experience lead to increased volume of subcompartments of the honeybee mushroom body. *Behavioral and Neural Biology* **62**:256-263.

Fahrbach SE, Robinson GE. 1995. Behavioural development in the honey bee: Toward the study of learning under natural conditions. *Learning and Memory* **2**:199-224.

Fahrbach SE, Robinson GE. 1996. Juvenile hormone, behavioral maturation, and brain structure in the honey bee. *Developmental Neuroscience* **18**:102-114.

Fahrenholz L, Lamprecht I, Schricker B. 1989. Thermal investigations of a honey bee colony: Thermoregulation of the hive during summer and winter and heat production of members of different bee castes. *Journal of Comparative Physiology B: Biochemical, Systemic, and Environmental Physiology* **159**:551-560.

Fluri P, Lüscher M, Willie H, Gerig L. 1982. Changes in weight of the pharyngeal gland and haemolymph titers of juvenile hormone, protein and vitellogenin in worker honey bees. *Journal of Insect Physiology* **28**:61-68.

Frings H. 1944. The loci of olfactory end-organs in the honey-bee, *Apis mellifera Linn*. *The Journal of Experimental Biology* **97**:123-134.

Gerber B, Wüstenburg D, Schütz A, Menzel R. 1998. Temporal determinants of olfactory long-term retention in honeybee classical conditioning: Nonmonotonous effects of the training trail interval. *Neurobiology of Learning and Memory* **69**:71-78.

Getz WM, Page RE. 1991. Chemosensory kin-communication systems and kin recognition in honey bees. *Ethology* **87**:298-315.

Giray T, Robinson GE. 2004. Effects of intracolony variability in behavioural development on plasticity of division of labour in honey bee colonies. *Behavioural Ecology and Sociobiology* **35**:13-20.

Giurfa M. 2003. Cognitive neuroethology: Dissecting non-elemental learning in a honeybee brain. *Current Opinion in Neurobiology* **13**:726-735.

Gould JL. 1974. Honey bee communication. *Nature* **252**:300-301.

Grosmaitre X, Marion-Poll F, Renou M. 2001. Biogenic amines modulate olfactory receptor neurons firing activity in *Mamestra brassicae*. *Chemical Senses* **26**:653-661.

Guzmán-Novoa E, Hunt GJ, Uribe JL, Smith C, Arechavaleta-Velasco ME. 2002. Confirmation of QTL effects and evidence of genetic dominance of honebee defensive behavior: Results of colony and individual behavioural assays. *Behavior Genetics* **32**:95-102.

Guzmán-Novoa E, Page RE. 1994. Genetic dominance and worker interactions affect honeybee colony defense. *Behavioural Ecology* **5**:91-97.

Harris JW, Woodring J. 1995. Elevated brain dopamine levels associated with ovary development in queenless worker honey bees (*Apis mellifera* L.). *Comparative Biochemistry and Physiology Part C: Pharmacology, Toxicology and Endocrinology* **111**:271-279.

Haydak MH. 1970. Honey bee nutrition. *Annual Review of Entomology* **15**:143-156.

Hölldobler B, Wilson EO. 1990. *The Ants*. Spronger-Verla, Heidelberg, Berlin.

Hölldobler B, Wilson EO. 2009. *The Superorganism: The beauty, elegance and strangeness of insect societies*. *W.W. Norton & Company Inc. New York*.

Huang ZY, Robinson GE. 1999. Social control of division of labor in honey bee colonies. *Information Processing in Social Insects,* Birkhäuser Verlag, Basel. 165-186.

Hunt GJ, Guzman-Novoa E, Fondrk MK, Page RE. 1998. Quantitative trait loci for honey bee stinging behavior and body size. *Genetics* **148**:1203-1213.

Jaycox ER. 1976. Behavioral changes in worker honey bees (*Apis mellifera* L.) after injection with synthetic juvenile hormone (*Hymenoptera: Apidae*). *Journal of the Kansas Entomological Society* **49**:165-170.

Johnson BR. 2010. Division of labor in honeybees: Form, function, and proximate mechanisms. *Behavioural Ecology and Socibiology* **64**:305-316.

Kalmus H, Ribbands CR. 1952. The origin of the odours by which honeybees distinguish their companions. *Proceedings of the Royal Society of London. Series B, Biological Sciences* **140**:50-59.

Kolmes SA, Winston ML. 1988. Division of labour among worker honey bees in demographically manipulated colonies. *Insectes Sociaux* **35**:262-270.

Langstroth LL. 1852. *Beehive*. United States Patent Office, Philadelphia, Pennsylvania.

Ledoux MN, Winston ML, Higo H, Keeling CI, Slessor KN, Conte Y. 2001. Queen pheromonal factors influencing comb construction by simulated honey bee (*Apis mellifera* L.) swarms'. *Insectes Sociaux* **48**:14-20.

Lenoir JC, Laloi D, Dechaume-Moncharmont F-X, Solignac M, Pham M-H. 2006. Intra-colonial variation of the sting extension response in the honey bee *Apis mellifera*. *Insectes Sociaux* **53**:80-85.

Lindauer M. 1953. Division of labour in the honeybee colony. *Bee World* **34**:63-90.

Maurizio A. 1950. The influence of pollen feeding and brood rearing on the length of life and physiological condition of the honeybee preliminary report. *Bee World* **31**:9-12.

Menzel R. 1968. Das gedächtnis der honigbiene für spektralfarben. *Zeitschrift für vergleichende Physiologie* **60**:82-102.

Menzel R. 1985. Learning in honey bees in an ecological and behavioral context. *Experimental Behavioral Ecology*, G.Fischer, Stuttgart. 55-74.

Menzel R. 1999. Memory dynamics in the honeybee. *Journal of Comparative Physiology A: Neuroethology, Sensory, Neural, and Behavioral Physiology* **185**:323-340.

Menzel R, Erber J. 1978. Learning and memory in bees. *Scientific American* **239**:80-87.

Menzel R, Giurfa M. 2001. Cognitive architecture of a mini-brain: the honeybee. *TRENDS in Cognitive Sciences* **5**:62-71.

Menzel R, Müller U. 1996. Learning and memory in honeybees: From behavior to neural substrates. *Annual Reviews Neuroscience* **19**:379-404.

Moore AJ, Breed MD, Moor MJ. 1987a. The guard honey bee: Ontogeny and behaviour variability of workers preforming a specialized task. *Animal Behaviour* **35**:1159-1167.

Moore AJ, Breed MD, Moor MJ. 1987b. Characterization of guard behavior in honeybees, *Apis mellifera*. *Animal Behaviour* **35**:1159-1167.

Morgan SM, Huryn VM, Downes SR, Mercer AR. 1998. The effects of queenlessness on the maturation of the honey bee olfactory system. *Behavioural Brain Research* **91**:115-126.

Moritz RFA, Hillesheim E. 1990. Olfactory discrimination between group odours in honey bees: Kin or nestmate recognition? *Insectes Sociaux* **37**:90-99.

Oldroyd BP, Thompson GJ. 2006. Behavioural genetics of the honey bee *Apis mellifera*. *Advances in Insect Physiology* **33**:1-49.

Omholt SW. 1988. Relationships between worker longevity and the intracolonial population dynamics of the honeybee. *Journal of Theoretical Biology* **130**:275-284.

Oster GF, Wilson EO. 1978. *Caste and Ecology in the Social Insects*. Princeton University Press, Princeton.

Painter TS, Biesele JJ. 1966. The fine structure of the hypophyaryngeal gland cell of the honey bee during development and secretion. *Proceedings of the National Academy of Sciences of the United States of America* **55**:1414-1419.

Pankiw T, Page RE. 1999. The effect of genotype, age, sex, and caste on response thresholds to sucrose and foraging behaviour of honey bees (*Apis mellifera* L.). *Journal of Comparative Physiology A: Neuroethology, Sensory, Neural, and Behavioral Physiology* **185**:207-213.

Pophof B. 2000. Octopamine modulates the sensitivity of silkmoth pheromone receptor neurons. *Journal of Comparative Physiology A: Neuroethology, Sensory, Neural, and Behavioral Physiology* **186**:307-313.

Reinhardt JF. 1952. Some responses of honey bees to alfalfa flowers. *The American Naturalist* **86**:257-275.

Rembold H. 1976. *The Role of Determinator in Caste Formation in the Honeybee*. Phase and Caste Determination in Insects. Pergamon Press, Oxford. 21-34.

Riley JR, Greggers U, Smith AD, Reynolds DR, Menzel R. 2005. The flight paths of honeybees recruited by the waggle dance. *Nature* **435**:205-207.

Robinson GE. 1987. Regulation of honey bee age polyethism by juvenile hormone. *Behavioural Ecology and Socibiology* **20**:329-338.

Robinson GE. 1992. Regulation of division of labor in insect societies. *Annual Revision of Entomology* **37**:637-665.

Robinson GE, Page RE. 1988. Genetic determination of guarding and undertaking in honey-bee colonies. *Nature* **333**:356-358.

Robinson GE, Page RE. 1989. Genetic determination of nectar foraging, pollen foraging, and nest-site scouting in honey bee colonies. *Behavioural Ecology and Socibiology* **24**:317-323.

Rossel S, Wehner R, Lindauer M. 1978. *E*-Vector orientation in bees. *Journal of Comparative Physiology A: Neuroethology, Sensory, Neural, and Behavioral Physiology* **125**:1-12.

Rothenbuhler WC. 1964. Behavior genetics of nest cleaning in honey bees. IV. Responses of F_1 and backcross generations to disease-killed brood. *American Zoologist* **4**:111-123.

Roussel E, Carcaud J, Sandoz J, Giurfa M. 2009. Reappraising social insect behavior through aversive responsiveness and learning. *Public Library of Science ONE* **4** e4197.

Scheiner R, Page RE, Erber J. 2001. Responsiveness to sucrose affects tactile and olfactory learning in performing honey bees to two genetic strains. *Behavioural Brain Research* **120**:67-73.

Schulz DJ, Robinson GE. 1999. Biogenic amines and division of labor in honey bee colonies: Behaviorally related changes in the antennal lobes and age-related changes in the mushroom bodies. *Journal of Comparative Physiology A: Neuroethology, Sensory, Neural, and Behavioral Physiology* **184**:481-488.

Schulz DJ, Vermilio MJ, Huang ZY, Robinson GE. 2002. Effects of colony food shortage on social interactions in honey bee colonies. *Insectes Sociaux* **49**:50-55.

Schwaerzel M, Monastirioti M, Scholz H, Friggi-Grelin F, Birman S, Heisenberg M. 2003. Dopamine and octopamine differentiate between aversive and appetitive olfactory memories in *Drosophila*. *The Journal of Neuroscience* **23**:10495-10502.

Seeley TD. 1979. Queen substance dispersal by messenger workers in honey bee colonies. *Behavioral Ecology and Sociobiology* **5**:391-415.

Seeley TD. 1982. Adaptive significance of the age polyethism schedule in honeybee colonies. *Behavioral Ecology and Sociobiology* **11**:287-293.

Seeley TD, Morse RA. 1976. The nest of the honey bee (*Apis mellifera* L.). *Insectes Sociaux* **23**:495-512.

Sekiguchi K, Sakagami F. 1966. Structure of the foraging population and related problems in the honeybee with considerations on the division of labour in bee colonies. *Hokkaido National Agricultural Experiment Station* **69**:1-65.

Slessor KN, Kaminski LA, King GGS, Borden JH, Winston ML. 1988. Semiochemical basis of the retinue response to queen honey bees. *Nature* **332**:354-356.

Smedal B, Brynem M, Kreibich CD, Amdam GV. 2009. Brood pheromone suppresses physiology of extreme longevity in honeybees (*Apis mellifera*). *The Journal of Experimental Biology* **212**:3795-3801.

Takeda K. 1961. Classical conditioned response in the honey bee. *Journal of Insect Physiology* **6**:168-179.

Tamer KG, Raphael B, Victoria S, Abraham H. 2006. Queen pheromones affecting the production of queen-like secretion in workers. *Journal of Comparative Physiology A: Neuroethology, Sensory, Neural, and Behavioral Physiology* **192**:737-742.

Tautz J. 1996. Honeybee waggle dance: Recruitment success depends on the dance floor. *The Journal of Experimental Biology* **199**:1375-1381.

Taylor DJ, Robinson GE, Logan BJ, Laverty R, Mercer AR. 1992. Changes in brain amine levels associated with the morphological and behavioural development of the worker honeybee. *Journal of Comparative Physiology A: Neuroethology, Sensory, Neural, and Behavioral Physiology* **170**:715-721.

Thom C, Gilley DC, Hooper J, Esch HE. 2007. The scent of the waggle dance. *Public Library of Science Biology* **5**:e228.

Unoki S, Matsumoto Y, Mizunami M. 2005. Participation of octopaminergic reward system and dopaminergic punishment system in insect olfactory learning revealed by pharmacological study. *European Journal of Neuroscience* **22**:1409-1416.

Uribe-Rubio JL, Guzman-Novoa E. 2008. Genotype, task specialization, and nest environment influence the stinging response thresholds of individual Africanized and European honeybees to electrical stimulation. *Behavioural Genetics* **38**:93-100.

Velarde RA, Robinson GE, Fahrbach SE. 2009. Coordinated response to developmental hormones in the Kenyon cells of the adult worker honey bee brain (*Apis mellifera* L.). *Journal of Insect Physiology* **55**:59-69.

Vereecken NJ, Cozzolino S, Schiest FP. 2010. Hybrid floral scent novelty drives pollinator shift in sexually deceptive orchids. *BioMed Central Evolutionary Biology* **10**:103-115.

Vergoz V. 2004. Conditionnement aversif chez l'abeille *Apis mellifera. DEA de neurosiences, comportement congnition Toulouse III France*.

Vergoz V. 2008. *Effects of Queen Mandibular Pheromone on Locomotor Behaviour and Learning in Worker Honey Bees, Apis mellifera*. In: Zoology. Dunedin: University of Otago.

Vergoz V, Roussel E, Sandoz J, Giurfa M. 2007a. Aversive learning in honeybees revealed by the olfactory conditioning of the sting extension reflex. *Public Library of Science ONE* **2**:e288.

Vergoz V, Schreurs HA, Mercer AR. 2007b. Queen pheromone blocks aversive learning in younger worker bees. *Science* **317**:384-386.

von Frisch K. 1967. *The Dance Language and Orientation of Bees*. Cambridge, Massachusetts: Harvard University Press.

Waddington KD, Gottlieb N. 1990. Actual vs. perceived profitability: A study of floral choice of honey bees. *Journal of Insect Behaviour* **3**:429-441.

Wagener-Hulme C, Kuehn JC, Schulz DJ, Robinson GE. 1999. Biogenic amines and division of labor in honey bee colonies. *Journal of Comparative Physiology A: Neuroethology, Sensory, Neural, and Behavioral Physiology* **184**:471-479.

Waller GD. 1972. Evaluating responses of honey bees to sugar solutions using artificial flower feeder. *Entomological Society of America* **65**:857-862.

Wehner R, Bernard GD, Geiger E. 1975. Twisted and non-twisted rhabdoms and their significance for polarization detection in the bee. *Journal of Comparative Physiology A: Neuroethology, Sensory, Neural, and Behavioral Physiology* **104**:225-245.

Wenner AM. 1962. Sound production during the waggle dance of the honey bee. *Animal Behaviour* **10**:79-95.

Wenner AM, Wells PH, Rohlf FJ. 1967. An analysis of the waggle dance and recruitment in honey bees. *Physiological Zoology* **40**:317-344.

Wildman TA. 1770. *A Treatise on the Management of Bees*. Wildman, T & Cadell, T. London.

Wilson EO. 1971. *The Insect Societies*. Belknap Press of Harvard University Press, Cambridge.

Wilson EO. 1985. The sociogenesis of insect colonies. *Science* **228**:1489-1495.

Winston ML. 1987. *The Biology of the Honey Bee*. Harvard University Press, Cambridge, Massachusetts.

Winston ML, Higo HA, Slessor KN. 1990. The effect of various dosages of queen mandibular gland pheromone on the inhibition of queen rearing in the honey bee (Hymenoptera: Apidae). *Annals of the Entomology Society of America* **83**:234-238.

Withers GS, Fahrbach SE, Robinson GE. 1995. Effects of experience and juvenile hormone on the organisation of the mushroom bodies of honey bees. *Journal of Neurobiology* **26**:130-144.

Wüstenberg D, Gerber B, Menzel R. 1998. Long- but not medium-term retention of olfactory memories in honeybees is impaired by actinomycin D and anisomycin. *European Journal of Neuroscience* **10**:2742-2745.

Zar JH. 1984. *Biostatistical Analysis*. Prentice Hall, Englewood Cliffs. N.J. 232-233.

7 Appendix A

Tables from the analysis out puts of the gradient decline of CS- responses in section 4.5 for all bee groups tested.

	Slope	**S.E.**	**t**	**Sig.**	**R^2**
2 Day olds	-3.667	1.293	-2.836	0.047	0.668
3 Day olds	-3.238	0.828	-3.911	0.017	0.793

Table 1. Coefficient outputs from linear regression of 2 & 3 day old bees' sting extension response to CS- during differential conditioning.

	Slope	**S.E.**	**t**	**Sig.**	**R^2**
Guard bees	-1.333	0.568	-2.347	0.079	0.579
Winter pollen foragers	-7.095	1.404	-5.054	0.007	0.865
Summer pollen foragers	-2.286	0.419	-5.458	0.005	0.882

Table 2. Coefficient outputs from linear regression from guards, winter and summer pollen foragers' sting extension response to CS- in differential conditioning.

	Slope	**S.E.**	**t**	**Sig.**	**R^2**
Summer pollen foragers	-2.286	0.419	-5.458	0.005	0.882
Pollen foragers, 10 minute ISI	-3.265	0.637	-5.125	0.007	0.868
Pollen foragers, 20 minute ISI	-3.714	0.444	-8.372	0.001	0.946

Table 3. Coefficient outputs from linear regression from pollen foragers' sting extension reflex to CS- based on time length between conditioning exposure.